# Development of an Optimised Cryopreservation Protocol for Encapsulated Liver Cell Spheroids: towards delivery of a Bioartificial Liver

A thesis submitted for the degree of Doctor of Philosophy (PhD)

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**Declaration**

I, Isobel Ruth Massie confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

**Abstract**

Acute liver failure (ALF) has a rapid and unpredictable onset with high mortality. The only current treatment is to transplant a donor organ but donor organ shortages exist. A bioartificial liver (BAL) could bridge the gap to transplant or buy time for spontaneous recovery. This BAL comprises HepG2 cells encapsulated within alginate that have formed tissue-like spheroids (ELS), which display upregulated function compared to monolayer cultures approximately 9 days after encapsulation.

In order to treat ALF in a timely fashion, this thesis aimed to provide an off-the-shelf treatment via development of a cryopreservation protocol for ELS.

A baseline recovery for ELS post-cryopreservation was established with particular attention paid to the clinically relevant time-course of recovery. Intracellular ice formation was identified as an injury mechanism causing large amounts of cell death. A strategy was devised to limit this and improved recovery was demonstrated.

Other, more subtle, injury mechanisms were subsequently identified and steps taken to ameliorate their effects resulting in decreased apoptosis and improved ELS recovery.

As ELS will be used to treat human patients as the cellular component of a BAL, a number of regulations must be complied with. The optimised cryopreservation protocol was modified to ensure mandates were met without a loss in ELS cell recovery.

Using this knowledge, cryopreservation was then scaled-up near to volumes that would be required to treat an adult patient.

Finally, these techniques were applied to cryopreservation of single cell suspensions of primary human hepatocytes. By doing so, it was determined whether or not a different cell type in a different culture format suffered injury via the same mechanisms as ELS and whether or not these could be ameliorated using the same methods.

In conclusion, an optimised cryopreservation protocol has been developed for ELS which may be used within a BAL.

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**Abbreviations**

3-D: 3-dimensional

7-EC: 7-Ethoxycoumarin

7-HC: 7-Hydroxycoumarin

Å: Angstrom

σ: Reflection co-efficient

Ab: Antibody

AAT: Alpha-1-antitrypsin

AFP: Alpha-1-fetoprotein

ALF: Acute liver failure

alpha-MEM: Alpha minimum essential medium

AO: Antioxidant

ATP: Adenosine triphosphate

BAL: Bioartificial liver

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

CIDOCD: Cryopreservation-induced delayed onset cell death

CPA: Cryoprotectant

CRF: Controlled rate freezer

CYP450: Cytochrome P450

DCF: Dichlorohydrofluorescein diacetate

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

DO: Dissolved oxygen

DSC: Differential scanning calorimetry

ECACC: European Collection of Cell Cultures

ECM: Extracellular matrix

ECOD: 7-EC-O-deethylation

EDTA: Ethylene diamine tetraacetic acid

ELAD: Extracorporeal liver assist device

ELISA: Enzyme-linked immunosorbent assay

ELS: Encapsulated liver cell spheroids

FBB: Fluidised bed bioreactor

FCS: Fetal calf serum

FDA: Fluorescein diacetate

FFP: Fresh frozen plasma

GMP: Good manufacturing practice

HBSS: Hanks balanced salt solution

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HG: High glucose

HGF: Hepatocyte growth factor

HRP: Horseradish peroxidise

HSP: Heat shock protein

IIF: Intracellular ice formation

IIP: Intraclellular ice propagation

IU: International units

J: Joules

K: Kelvin

KPS: Kidney perfusion solution

LDH: Lactate dehydrogenase

LG: Liver group

$L_p$: Membrane permeability to water

MARS: Molecular adsorbent recirculating system

MDA: Malondialdehyde

MeOH: Methanol

MF: Multiplication factor

MTT: Methylthiazolyldiphenyl-tetrazolium bromide

NIBSC: National Institute for Biological Standards and Control

NS: Not significant

OLT: Orthotopic liver transplant

OPD: O-phenylenediamine

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate buffered saline

PERV: Porcine endogenous retrovirus

PFC: Perfluorodecalin

PG: Propylene glycol

PHH: Primary human hepatocyte

PI: Propidium iodide

P/S: Penicillin/streptomycin

RCCS: Rotating cell culture system

ROCK: Rho-associated kinase

ROS: Reactive oxygen species

rpm: Revolutions per minute

RT: Room temperature

ST: Staurosporine

TBA: Thiobarbituric acid

TBARS: Thiobarbituric acid reactive substance

TCA: Trichloroacetic acid

TNFa: Tumour necrosis factor-alpha

TRH: Thyroid releasing hormone

UV: Ultraviolet

UW: University of Wisconsin

WEM: William's medium E

**Acknowledgements**

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Last but certainly not least, a huge thank you to Tom, my family and friends who have been nothing but supportive and encouraging. I cannot thank you all enough.

# CHAPTER 1

# Introduction

Despite improved treatment regimes, mortality of acute liver failure (ALF) remains high in Europe, the US and Japan (Ichai & Samuel 2011). Currently, the only recognised treatment is orthotopic liver transplant. Unfortunately, due to donor organ shortages and the logistics of being able to deliver a suitable organ within a suitable time frame to the patient, many die waiting for a liver transplant (Nguyen & Vierling 2011). However, this mortality could be reduced using a bioartificial liver device (BAL). A BAL is intended as a temporary bridge to either transplant or spontaneous recovery but must replace healthy liver function and must be available for unpredictable emergency use. The following study investigates methods for cryopreservation of alginate-encapsulated HepG2 3D cell spheroids (ELS) as the cellular component of a BAL.

This chapter will give an overview of:

- the structure and functions of the liver
- hepatic failure and its management
- cryopreservation principles
- *in vitro* methods to measure success following cryopreservation

## 1.1. Introduction to the liver

The liver is the largest internal organ, weighing approximately 1.5kg in adults. It performs a number of wide-ranging functions including metabolism and synthesis (Moore & Agur 2007).

### 1.1.1. Structure of the liver

The liver is located in the upper right quadrant of the abdomen. It is bisected by the falciform ligament which divides the liver into 2 functionally independent lobes. Each lobe has a dual blood supply. Each lobe is further separated into lobules that are typically hexagonally-shaped (see Figure 1-1). The hepatic artery supplies oxygen-rich blood whilst the hepatic portal vein supplies nutrient- and toxin-rich blood from the spleen, pancreas and gastrointestinal tract. These blood supplies drain into sinusoids between the cells of the liver allowing them to be perfused with blood, which is then processed by the liver, before leaving through the hepatic vein. Most waste from the

liver is removed via the hepatic bile duct which joins with the cystic duct (from the gall bladder) to form the common bile duct which then empties into the duodenum. Other waste is removed from the liver via blood where it is transported to the kidneys for excretion as urine (Moore & Agur 2007).

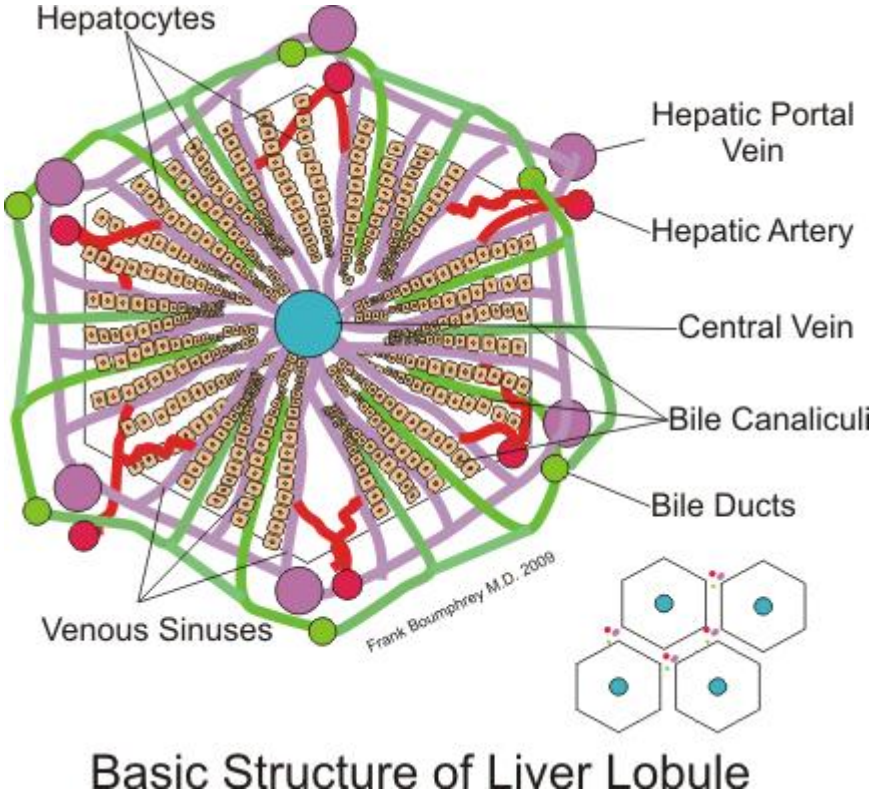


**Figure 1-1. Structure of liver lobule.**

**Liver lobules are classically considered to be hexagonal. The portal triad (hepatic portal vein, hepatic artery and bile duct) is located at the vertices of the lobule. The central vein is at the centre of the lobule. Blood flows from the portal triad to the central vein. Bile flows out through the hepatic bile duct (Taken from "Hepatic Structure" 2011).**

### 1.1.2. Cells of the liver

The liver contains many different cell types that are required for normal liver function. These include hepatocytes, Kupffer cells, stellate cells and oval cells.

#### 1.1.2.1. Hepatocytes

Hepatocytes comprise approximately two thirds of the liver cell population and are responsible for the majority of liver function. Morphologically, they normally appear cuboidal with a large, central nuclei. Hepatocytes may be tetraploid and binuclear cells are common. Hepatocytes are extremely metabolically active and this is reflected by high numbers of mitochondria contained within their cytoplasm. They also contain

large amounts of rough and smooth endoplasmic reticulum (for synthesis and transport of proteins and lipids), lysosomes, peroxisomes and endocytic vesicles (for digestion), storage vacuoles, glycogen vacuoles and fat droplets. Hepatocytes also display a unique capability for regeneration following disease or injury. In liver acini, hepatocytes form plates one cell thick and approximately 20 cells long that run between the portal triad and hepatic vein. Sinusoids separate these plates and perfuse the cells with blood (Read 1972).

#### 1.1.2.2. Kupffer cells

Kupffer cells are normally located at the lining of the sinusoids and are macrophages responsible for phagocytosis (of red blood cells) and cytokine production (including tumour necrosis factor alpha (TNFa) (Tacke et al. 2009).

#### 1.1.2.3. Stellate cells

Stellate cells are found within the Space of Disse (between hepatocytes and sinsusoids). Quiescent stellate cells store fat droplets and retinoic acid. However, upon activation by TNFa, they produce growth factors and extracellular matrix which are involved in liver regeneration and fibrosis (Kordes et al. 2009; Sato et al. 2003).

#### 1.1.2.4. Oval cells

Oval cells are found in or near bile ducts of Canals of Hering (bile ductules). Although the exact properties of oval cells are not agreed upon, it is generally accepted that they are bipotential stem cells with the potential to differentiate into either hepatocytes or cholangiocytes (bile duct epithelial cells). They are phenotypically similar to the hepatoblasts that are required for liver development and activated in response to growth factors released following disease or injury when mitosis of hepatocytes cannot occur (Bird et al. 2008; Oertel & Shafritz 2008).

### 1.1.3. Functions of the liver

As mentioned above, the functions of the liver are performed by hepatocytes. These functions include synthesis, metabolism and detoxification.

#### 1.1.3.1. Synthesis

Hepatocytes synthesise and secrete numerous proteins such as haem and plasma proteins. Plasma proteins produced include clotting factors, such as fibrinogen and

prothrombin, and acute phase proteins, but also albumin. Albumin is the most abundant plasma protein and is responsible for maintenance of oncotic pressure and also binding lipid-soluble toxins for excretion. Hepatocytes also synthesise bile salts that are required for emulsification and digestion of lipids and hydrophobic vitamins (Moore & Agur 2007).

#### 1.1.3.2. Metabolism

The liver maintains blood glucose levels via 3 methods: gluconeogenesis (synthesis of glucose from amino acids, glycerol and lactate); glycogenolysis (breakdown of glycogen to glucose); and glycogenesis (formation of glycogen from glucose). The latter 2 are regulated by glucagon and insulin respectively.

The liver also breaks lipids down into their component parts and performs lipogenesis (fatty acid and triglyceride synthesis). The products of this process are excreted from the liver as lipoproteins. The liver also produces cholesterol, essential for plasma membrane fluidity and permeability.

The liver is also responsible for transamination (amino acid conversion). Non-essential amino acids are produced in this way. Also, any excess essential amino acids may be converted to produce potentially toxic ammonia and amino groups (Moore & Agur 2007).

#### 1.1.3.3. Detoxification

Ammonia is a normal by product of transamination but its build-up (hyperammonaemia) can result in encephalopathy. The liver detoxifies ammonia by converting it to urea by completion of the urea cycle. Urea is then excreted as part of urine by the kidneys.

Xenobiotics are also detoxified in the liver by a multistep process. Phase I reactions, typically oxidation by cytochrome P450 enzymes, convert xenobiotics into polar moieties. Phase II reactions then detoxify these metabolites by reaction with the polar functional group. Phase III reactions may be required after this before the metabolites and by-products are excreted (Moore & Agur 2007).

## 1.2. Acute liver failure

Clinically, ALF is defined as the rapid (less than 8 weeks but usually shorter) onset of liver failure with diminished mental function and coagulopathy resulting from the

sudden loss of up to 90% liver function. Onset is unpredictable and rapid and is associated with a high mortality (Nguyen & Vierling 2011).

### 1.2.1. Causes

ALF may be caused by toxic insult such as alcohol or paracetamol overdose, or less commonly mushroom poisoning. Alternatively, chronic liver damage resulting from persistent alcohol abuse leading to fibrosis may cause sufficient cirrhosis that the condition becomes acute. Viral infections, including Hepatitis A and B, may also lead to ALF. Similarly, some autoimmune diseases and Wilson's disease (causing copper accumulation and subsequent liver damage) may result in ALF (Ichai & Samuel 2011; Nguyen & Vierling 2011).

### 1.2.2. Symptoms

ALF has a multitude of symptoms, reflecting the wide range of functions normally performed by the liver. Patients typically present with jaundice (due to an accumulation of unconjugated bilirubin in the blood) abnormal blood clotting (as clotting proteins are not being synthesised), and alterations in mental status (due to toxin and ammonia build up). This typically progresses to encephalopathy and brain herniation. Furthermore, 50% of patients will also suffer renal failure due to failure to manage blood pressure correctly and up to 80% patients will also suffer opportunistic infections. Finally, multisystem organ failure is common (Gerlach et al. 2008).

### 1.2.3. Treatment

The only proven current treatment for ALF is orthotopic liver transplant (OLT). With OLT, mortality is reduced to around 30%. Donor organs may be sourced from deceased or living donors (Craig et al. 2009). There is also a cohort of patients with ALF who show spontaneous recovery but this cannot be predicted. If these patients could be supported through ALF (using a BAL), unnecessary transplantation could be avoided.

### 1.2.4. Management

#### 1.2.4.1. Intensive care

Patients with ALF may recover spontaneously and so their condition may be managed using intensive care treatments. Ventilation may be required, and plasmapheresis or plasma exchange used to remove toxins in the blood and replace liver proteins.

Complications such as hypoglycaemia and acidosis may be treated using dextrose or bicarbonate respectively (Polson & Lee 2005). Mortality of ALF patients treated in this way remains high at approximately 75% although some estimates are as high as 90% depending on aetiology.

#### 1.2.4.2. Artificial liver devices

Artificial livers are devices that aim to bridge the gap either to transplant or spontaneous recovery. These devices typically rely on filtration and/or sorbents to remove toxins from ALF patients' plasma. Generally, these devices have been shown to improve some biochemical parameters but no significant extension in survival time has been demonstrated in an appropriate clinical trial as yet. The reasons behind this are likely to be that removal of toxins is non-specific and that the synthetic and metabolic functions of the liver are not performed.

##### *1.2.4.2.1. Prometheus*

The Prometheus (Fresenius Medical Care, Sweden) works on the principle of filtration. First, blood is separated into plasma and cellular constituents. Plasma is filtered through 2 adsorber filters that separate toxins from albumin and bind them. The blood cells and plasma are then recombined and passed through a polysulfane high flux dialyser that removes hydrophilic toxins from blood. The "cleaned" blood is then returned to the patient. 11 patients were subjected to 50 treatments with the Prometheus and statistically significant improvements in serum unconjugated bilirubin, bile acids, creatinine, blood pH, urea and ammonia levels were observed. However, as one would expect, serum protein levels remained unchanged and 8 out of the 11 patients died (Rifai et al. 2003). More recently, a trial using patients with acute-on-chronic liver failure did not show any improvement in recovery compared with conventional medical therapy (Rifai et al. 2010).

##### *1.2.4.2.2. Molecular adsorbent recirculating system*

The molecular adsorbent recirculating system (MARS) is based upon the use of an albumin diasylate to remove toxins from ALF plasma across a semi-permeable membrane. Again, "cleaned" plasma is returned to the patient. In similar trials, 10 patients were treated for 2 sessions with MARS and statistically significant reductions in blood creatinine and urea were observed but ultimately 7 out of 10 patients died (despite 2 receiving OLT) (Lai et al. 2005). In a small (18 acute-on-chronic liver failure

patients) randomised control study, MARS was compared to standard medical treatment and MARS was shown to significantly reduce serum bilirubin, model for end stage liver disease score and encephalopathy grade. Survival was unaffected (Sen et al. 2004). A recent larger trial in acute-on-chronic liver failure also failed to show any improvement in mortality with this device (Banares et al. 2010).

1.2.4.3. Bioartificial liver devices (BALs)

BALs comprise a cellular component, hepatocytes, the parenchymal cells of the liver. The concept is that the entire range of liver function (synthesis, detoxification and metabolism) may be temporarily performed by extracorporeal hepatocytes contained within a BAL. The major difference between different BALs is the cell source utilised.

*1.2.4.3.1. HepatAssist*

The HepatAssist (Circe Medical, US) relies upon a charcoal filter to adsorb some toxins and $7x10^{10}$ porcine hepatocytes contained within a hollow-fibre column comprise the cellular component. Porcine hepatocytes have the advantage of being available in sufficient number, but carry a risk of viral transmission, in particular porcine endogeneous retroviruses (PERV). PERV are closely related to leukaemia and immunodeficient viruses and transmission to human cells has been demonstrated *in vitro.* However, transmission has not been observed *in vivo* either due to innate adaptive immunity or the inclusion of membranes between cells and patients designed to act as a physical barrier to transmission (Chamuleau et al. 2005). The HepatAssist has been used in a large (171 patients) randomised controlled study for treatment of ALF. Reduction in serum bilirubin was observed in the HepatAssist group cf. the standard medical treatment group. No significant improvement in survival was demonstrated overall; although in a subsequent sub-group analysis, a significant improvement in survival was demonstrated for fulminant/sub-fulminant hepatic failure patients (Demetriou et al. 2004).

*1.2.4.3.2. Extra-corporeal liver assist device*

The Extra-corporeal liver assist device (ELAD) (Vital Therapies, US) uses C3A cells as the cellular component housed within a hollow-fibre cartridge. C3A cells are a subclone of the HepG2 cell line. The advantage of utilising a cell line is that availability is sufficient and that cells are allogeneic. However, there is a risk of tumourigenesis. Despite this, the ELAD has undergone a trial, setting a regulatory

precedent. 24 patients were included in a pilot-controlled trial and although no significant differences between standard medical treatment and ELAD treatment were observed, safety was demonstrated (Ellis et al. 1996). More recent studies, using a revised geometry and cell number, have been performed, and this is currently undergoing trial in the US and Europe.

## 1.3. The Liver Group BAL

### 1.3.1. Cell source

The Liver Group (LG) BAL uses the HepG2 cell line to comprise the biomass. HepG2 cells are hepatoblastoma-derived and are extremely well characterised. They were originally isolated from a liver biopsy from a 15-year old Caucasian male. They possess the ability to synthesise many of the secreted proteins of primary hepatocytes (Slany et al. 2010) but do not possess comparable detoxification function of ammonia (Mavri-Damelin et al. 2007) or xenobiotics (Hart et al. 2010) compared to adult hepatocytes.

### 1.3.2. Alginate encapsulation

In the LG BAL, HepG2 cells are encapsulated within alginate. Alginate is a biocompatible polymer purified from seaweed and is a copolymer of mannuronic and guluronic acids. Alginate can be dissolved in water to form a hydrogel. This hydrogel acts as a scaffold onto which HepG2 cells can anchor. Alginate hydrogel is approximately 99% water which means that diffusion of nutrients and oxygen to the cells and diffusion of waste products away from the cells are possible allowing cell proliferation. Furthermore, unlike some other alginate-encapsulated culture formats, the alginate beads are not encased in poly-L-lysine (commonly employed to prevent immune responses) so that diffusion of the larger liver-secreted proteins is not inhibited (Orive et al. 2006).

The alginate matrix provides a scaffold to which HepG2 cells can adhere. Once attached, HepG2 cells proliferate at a rapid rate (faster than in monolayer cultures). This means that the large cell numbers required to treat ALF may be achieved more rapidly. Furthermore, using transmission electron microscopy, cell-cell contacts such as gap junctions and desmosomes may be observed. In addition, large amounts of extracellular matrix are visible, indicating that alginate encapsulation provides an environment for the HepG2 cells that mimics that which would be found *in situ*. These

tissue-like encapsulated liver cell spheroids (ELS) which begin to form after 2-3 days of culture demonstrate upregulated per cell function when compared to monolayer cultures after 8-9 days of culture. At this time each alginate bead contains approximately 20-25 spheroids, and each spheroid contains approximately 25 cells.

### 1.3.3. Fluidised bed bioreactor culture

Although beads can be cultured in static format, and indeed were for the majority of the work described in this thesis, culture within a microgravity or fluidised bed bioreactor (FBB) can be used to further increase proliferation rates (Coward et al. 2005) and this method would eventually be used to culture the biomass for the LG BAL. FBB culture allows better control of culture conditions, including oxygen content and pH and mass transfer is much improved. Upregulation of proliferation is desirable for 2 reasons. Firstly, as large numbers (estimated to be in the region of $7x10^{10}$) of functioning hepatocytes are required to treat ALF, this can be achieved more rapidly using FBB culture. Secondly, this method of culture allows cell density to be increased, facilitating use within a clinical setting where more than $10^{10}$ cells may be required to support a patients' liver function. Cells cultured in this way maintain function within liver failure plasma (Coward et al. 2009).

### 1.3.4. Liver Group bioartificial liver principle

In order to treat ALF, 3 stages must be completed. Firstly, individual HepG2 cells must be encapsulated within alginate. Then the cells must be cultured to form ELS. Finally, ELS must be perfused against the ALF patient's plasma.

The LG BAL comprises 2 extracorporeal circuits. In the first circuit, plasma would be separated from the remainder of the blood. The plasma would then flow round the second circuit where it would be perfused against ELS contained within a chamber. ELS would simultaneously detoxify plasma and secrete liver-specific proteins into the patient's plasma. The plasma would then return to the patient via a system designed to filter out cell debris and DNA, endotoxin and alginate particulates, thereby meeting regulatory requirements.

## 1.4. Biopreservation for BAL as short- or long-term strategies

The onset of ALF is extremely rapid and unpredictable, meaning an off-the-shelf treatment is highly desirable. For ELS to reach performance-competency (i.e. a

sufficient number of cells with sufficient upregulated function) takes between 8 and 9 days. Therefore, a method of preservation (either short-term at temperatures between 0 and 37°C or long-term at cryogenic temperatures) below the glass transition temperature is required.

### 1.4.1. Short-term preservation

Short-term storage of ELS could be used to deliver the biomass for a BAL as required on demand. This would mean that ELS could be batch produced and stored at hypothermic, although not cryogenic temperatures, for a pre-determined time until needed. Hypothermic storage is typically in the region of 4°C. Clearly, cold-chain is more feasible at this temperature and there is no requirement for liquid nitrogen or other cryogen, making this an attractive option. Current applications of storage at hypothermic temperatures include delivery of donor organs destined for transplantation and tissue-engineered constructs for regenerative medicine applications. However, this approach is limited to a few days at most.

#### 1.4.1.1. Organ preservation

As with other organs, such as kidneys or pancreases, the liver is subject to ischaemia/reperfusion injury following hypothermic storage. The ischaemia arises as the explanted organ is not sufficiently oxygenated during removal and storage thereafter. Ischaemia results in a shift away from aerobic to anaerobic metabolism. Reperfusion injury occurs as the organ is returned to 37°C and oxygen delivery is restored. Ischaemia/reperfusion injury can result in perfusion failure, initiation of inflammatory responses and oxidative stress, eventually leading to cell death and reduced graft or organ functionality (de Rougemont et al. 2009). Preservation solutions are used to flush the vascular bed of the organs and to cool them. University of Wisconsin (UW) solution is currently considered the gold standard for liver preservation (Feng et al. 2007).

Strategies to limit or reduce this injury include preconditioning steps to protect against the injury mechanisms described. These mechanisms are likely to be complex and varied although ischaemic preconditioning has been shown to reduce ischaemic injury in some studies (de Rougemont et al. 2009). This is likely to be effective as cells are stimulated to protect themselves against oxidative stress and maintain mitochondrial function without the need to determine the precise underlying cause (Tapuria et al.

2008). Despite these advances, function of such organs is not likely to be sustained after more than a few hours of hypothermia.

1.4.1.2. Tissue-engineered construct preservation

Much effort has been put into developing methods for effective hypothermic storage of tissue-engineered constructs, notably tissue-engineered skin. Tissue-engineered skin is often required to be available off-the-shelf to treat burns and ulcers, for example, where rapid treatment is necessary to prevent dehydration and infection. Using an appropriate preservation media, hypothermic storage of a tissue-construct comprising gel-embedded keratinocytes is possible for up to 1 week (Cook et al. 1995).

1.4.1.3. Storage at intermediate temperature with oxygenated support (perfluorodecalins)

Organs destined for transplantation may be transported with oxygenated support, typically in the form of perfluorodecalins (PFC). PFC can dissolve large amounts of oxygen to provide an additional supply of oxygen during transport (Baertschiger et al. 2008). PFC is approximately twice as dense as water and so it is possible to store organs using the two-layer-method; organs may be sandwiched between a layer of PFC and organ preservation solution (Liu et al. 2007).

### 1.4.2. Long-term cryopreservation

Long-term storage of ELS is preferable to short-term preservation for many reasons. Firstly, ELS could be produced on a very large scale and cryopreserved in single-treatment aliquots for thawing as and when required for emergency treatment. This would also mean that confidence of all ELS aliquots produced in that run could be guaranteed to the clinic. Additionally, wastage would be reduced as the shelf-life would be far longer than ELS stored at or around 4°C. Cryopreservation allows storage at deep cryogenic temperatures, where all molecular interactions are inhibited, and storage for months or years can be assured. For example sperm have been successfully stored for more than 20 years (Yogev et al. 2010). Furthermore, there is less pressure on the ELS production runs themselves; if there were a problem (e.g. infection of the biomass) during production, a longer shelf-life and back-up supplies would provide far more flexibility and treatment could still be provided from previously manufactured and stored ELS.

Cryopreservation is routinely and relatively successfully used to store various cell types, in particular gametes and embryos, but usually as single cell suspensions and in small volumes. Cryopreservation of larger tissues, including corneas, cartilage and arteries, has also been attempted but with mixed success (Muldrew et al. 2000; Pegg 2006; Wusteman et al. 1997). The principles of cryopreservation, though, are well understood and will be described in the following section. Far less is known about cryopreservation of inter-connecting multicellular structures, such as ELS, or cryopreservation of large-scale volumes as would eventually be required for treatment of ALF.

### 1.4.3. Cooling rates during cryopreservation

Each cell type has a unique optimal cooling rate that should be applied during cryopreservation for optimal recovery in post-thaw cultures. This is demonstrated in Figure 1-2 below. The range of optimal cooling rates is very large and, for example, ranges from 3°C/min for stem cells to 3000°C/min for red blood cells (Mazur 2004). The optimal cooling rate reflects avoidance of 2 related injury mechanisms: solute toxicity and intracellular ice formation.

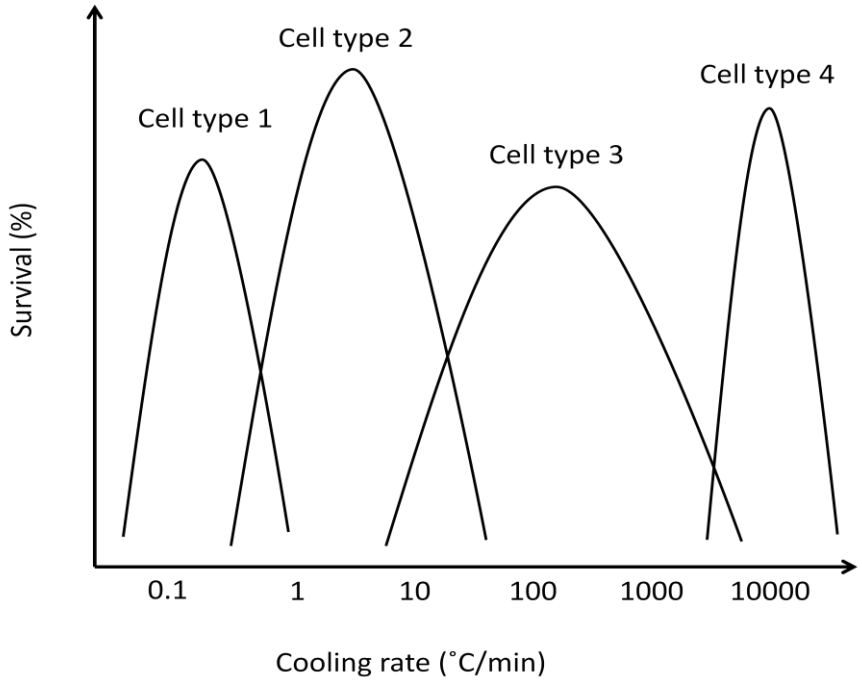


**Figure 1-2. The "inverted U".**

**Each cell type has a unique optimal cooling rate. The range of cooling rates is very large and can differ by 5 orders of magnitude.**

1.4.3.1. Cell death at slow cooling rates – solute toxicity

At slow cooling rates, ice will consistently nucleate and form outside the cell. As the ice forms, solutes are excluded from the ice crystals, which increases the concentration of these solutes in the remaining unfrozen water fraction. This increase in concentration results in cell dehydration as water leaves the cell by osmosis to regain equilibrium with the external solution. In turn, the concentration of solutes inside the cell will increase.

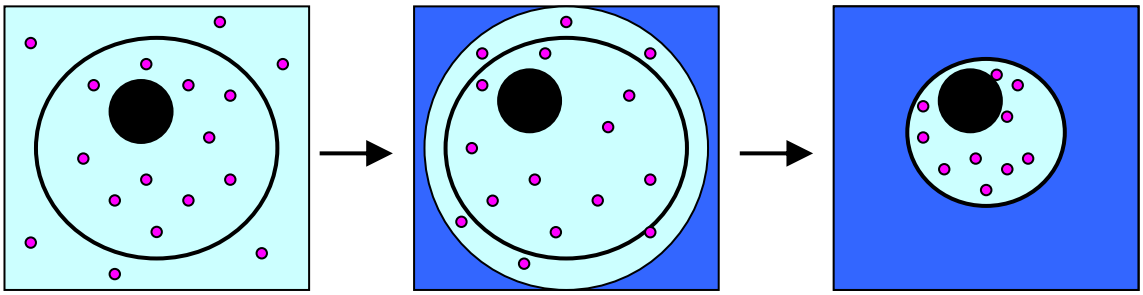

**Figure 1-3. Mechanism of cell injury during slow cooling.**

**Cell in unfrozen solution (left). Ice formation (dark blue) occurs outside the cell which increases solute (pink) concentration in remaining unfrozen fraction (middle). Cell dehydrates by osmosis to retain equilibrium, increasing solute concentration inside the cell (right) leading to solute toxicity.**

The mechanisms that eventually lead to cell death from cooling at too slow a rate remain to be fully defined. However, it is likely that severe dehydration may lead to membrane destabilisation and blebbing, irreversibly comprising plasma membrane integrity. Where efflux of water from the cell occurs very rapidly, shear stresses exerted on the cell membrane may be sufficient to rupture the membrane, rendering the cells membrane-permeabilised. Furthermore, exposure to hypertonic solutions is likely to be associated with disruption to the biochemical and physical conditions needed for cell survival (Dumont et al. 2004; Kleinhans 1998; Muldrew et al. 2004). Ultrastructural damage to organelles, particularly mitochondria, can also have serious negative effects on recovery post-cryopreservation.

Slow-cooling injury has also been suggested to cause apoptosis in post-thaw cultures as the cells respond to stresses associated with the cryopreservation regime. These stresses include osmotic (upon addition of CPA and ice formation), hypothermia, oxidative stress and energy depravation which occur during cryopreservation. However, the resultant apoptotic response is often not apparent until up to 12 hours post-warming.

Baust et al. describe this apoptotic response more fully (Baust et al. 2009). The effect of these stresses on ELS and primary human hepatocytes will be discussed more fully in Chapters 4 and 7 of this thesis respectively.

1.4.3.2. Cell death at rapid cooling rates

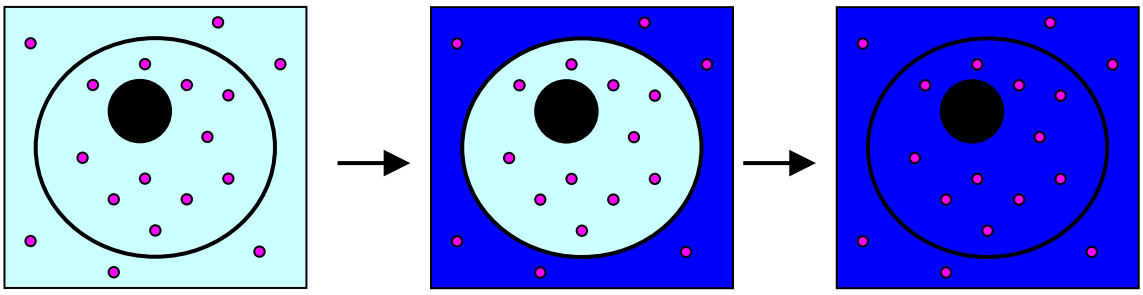

**Figure 1-4. Mechanism of cell injury during rapid cooling.**

**Cell in unfrozen solution (left). Ice formation (dark blue) occurs outside the cell which cannot dehydrate on a kinetic basis (middle) resulting in intracellular ice formation (right).**

At rapid cooling rates, ice will again form outside the cell. However, in this instance, the cells are unable to dehydrate on a kinetic basis and mobile water persisting inside the cells permits intracellular ice formation (IIF) (Mazur & Koshimoto 2002). This is represented in Figure 1-4 above. IIF does not necessarily cause cell death. It has been shown that cells are able to withstand IIF if the volume of ice is not too great and ice crystals are sufficiently small (Karlsson et al. 1993; Muldrew et al. 2004). However, in reality, IIF often results in cell death (Mazur 1961). This occurs as a result of mechanical disruption of both plasma membranes and other cell organelles and protein denaturation.

*1.4.3.2.1. Membrane transport*

The optimal cooling rate for each cell type was traditionally determined experimentally. However, mathematical modelling has also been used to predict and/or determine which cooling rates should be used for which cell type. The "inverted U" graph demonstrates that the 2 injury mechanisms are related as the 2 injury mechanisms never overlap over the same temperature range, meaning that an "optimal" rate of cooling is always observed (Muldrew et al. 2004).

This is attributed to membrane transport characteristics, specifically those of water and, to a lesser degree, cryoprotectant. A cell's ability to dehydrate depends upon the permeability of the membrane to water, $L_p$ and the surface area to volume ratio. Water

is able to leave the cell via aqueous pores, known as aquaporins, which have small diameters of approximately 8Å or may simply diffuse across the plasma membrane (Mazur 1961). Various models exist to describe this but the most widely recognised is that of the two-parameter model (Mazur et al. 1974; Mazur & Miller 1976). Co-transport of water and solutes may occur and an additional term, σ (reflection co-efficient), is sometimes introduced (Kedem & Katchalsky 1958) but the effect of this is considered negligible and results from both models often yield very similar results (Kleinhans 1998). Generally, cells with high $L_p$ and low volumes with relatively high surface areas should be cryopreserved using rapid cooling rates and *vice versa*.

Human hepatocytes are relatively large cells (in the region of 20µm diameter) and as such possess relatively low $L_p$ (equivalent to approximately 2µm/min atmosphere) (Darr & Hubel 1997). For comparison, canine erythrocytes possess an $L_p$ of approximately 20µm/min atmosphere (Liu et al. 2002). However, variations in $L_p$ exist not only between species but also between primary cells and cell lines of the same type. For example, HepG2 cells possess a lower $L_p$ (0.2µm/min atmosphere) (Darr & Hubel 1997). Hydraulic conductivity also varies between between individual cells and those comprising multicellular aggregates (Korniski et al. 1999).

#### 1.4.3.3. Supercooling

Supercooling occurs when a liquid cools to below its equilibrium melting point without forming a solid. In itself, this is not damaging to cells. However, when freezing (nucleation) does occur, latent heat of crystallisation is released (as nucleation is an exothermic event). Again, this in itself is not necessarily damaging unless it occurs to a large degree. In this instance, cells are exposed to cooling rates greater than intended. The more the solution outside the cell supercools, the more exothermic the reaction is the greater is the difference between the sample (which is delayed from cooling by the nucleation reaction) and chamber temperature (which continues to cool with the applied cooling profile). Once nucleation is complete, the sample temperature will resume falling but at an increased rate to reach the chamber temperature, increasing the chance of IIF (Diller 1975; Zavos & Graham 1983). The supercooled state is metastable and will result in ice nucleation with probability of nucleation increasing with time held at the supercooled temperature.

##### *1.4.3.3.1. Methods to avoid supercooling*

Supercooling of samples is a commonly reported problem. As such, methods of inducing spontaneous nucleation have been developed to avoid/limit it. The most commonly used method is that of "seeding", which describes the introduction or production of a seed ice embryo, enabling propagation of ice throughout the remainder of the sample. Cryogen-cooled forceps may be used to cause deep localised cooling at the edge of the sample. This localised drop in temperature triggers ice nucleation which permits crystallisation throughout the remainder of the sample which is cooled to just below the equilibrium melting temperature. Provided that supercooling is not too great (i.e. the temperature of the bulk of the sample does not fall further than a few degrees below the equilibrium melting temperature) this can be very effective. "Seeding" is routinely recommended for cryopreservation of small samples, notably spermatozoa (Songsasen & Leibo 1997) and oocytes (Stachecki & Cohen 2004). However, this approach may be difficult to apply to larger sample volumes where heat transfer is restricted.

Alternatively, the application of a rapid cooling ramp may be used to "thermally shock" the sample into nucleation. Again this works by producing a temperature gradient throughout the sample to trigger nucleation (Zhang et al. 1996). This approach has successfully been applied to cryopreservation of hepatocytes (Diener et al. 1993; Terry et al. 2006). Again though, this approach mandates that the sample must be able to be shock cooled which is difficult to achieve when dealing with large sample volumes.

The final option is that of heterogeneous nucleators. Nucleation of water into ice can be either hetero- or homogeneous. Homogeneous nucleation of water occurs at very low temperatures in the region of -40°C. More often, nucleation is heterogeneous. Heterogeneous nucleation requires the presence of another material on the surface of which ice nucleation may occur at higher temperatures, closer to the equilibrium melting point. Heterogeneous nucleators have differing efficacies based upon how similar their surface topography or solid crystal surface is to that of ice. Where lattice size and types are similar, nucleation may occur readily at or slightly below the equilibrium melting point (the temperature of which nucleation becomes thermodynamically preferable). This approach is useful for larger samples where control of cooling rate is more difficult as the nucleators could be dispersed throughout the entire volume. Heterogeneous nucleators are common in nature where insects (Mugnano et al. 1996; Zachariassen & Hammel 1976) and some bacteria (Kawahara

2002) have adapted to the cold environment by producing ice nucleators. In turn, nucleators have been included during cryopreservation protocols for a variety of biologics such as fungal starters (Missous et al. 2007), embryos (Kojima et al. 1988) and spermatozoa (Chen et al. 1993) to trigger nucleation.

### 1.4.4. Vitrification

Vitrification can provide an alternative method for cryopreservation that avoids crystallisation of water to form ice entirely. Instead high concentrations of cryoprotectants (CPAs) (typically in the region of 40% v/v) and rapid cooling rates are used to result in formation of a "glassy" matrix. Technically this matrix is still a liquid, albeit an extremely viscous one, but the low temperature and high viscosity makes this a suitable method for long-term storage of cells and tissues (Fahy et al. 1984).

Vitrification has been used for successful cryopreservation of various cells including microencapsulated hepatocytes (Wu et al. 2007), embryos and oocytes (Kuleshova & Lopata 2002). However, it is restricted to samples where rapid cooling rates can be applied (i.e. small volumes) which makes its applicability, using traditional protocols, to large volumes of ELS for a BAL doubtful.

#### 1.4.4.1. Liquidus-tracking

Liquidus-tracking is a relatively new methodology that allows delivery of high concentrations of CPA without the need for rapid cooling rates but still resulting in vitrification. The liquidus temperature is defined as the highest temperature at which crystals can exist. Above the liquidus, no crystal (solid) can exist. The liquidus curve arises as with increasing solute concentration, the melting temperature decreases. Therefore, liquidus-tracking describes the technique of increasing solute (in this instance CPA) concentration whilst decreasing the temperature such that solidification never occurs but high concentrations of CPA may be delivered (Pegg et al. 2006). Cytotoxicity at these concentrations would normally be high but due to the low temperatures at which they are applied, it is assumed that this is not evident due to the cell's decreased activity.

Liquidus-tracking has been applied to cryopreservation of cartilage with good success (Pegg et al. 2006) although it is recognised that a complex experimental setup is required. Steps have since been made to simplify and automate the protocol, increasing ease of use within a laboratory setting (Wang et al. 2007).

### 1.4.5. Thawing/warming protocols

The thawing protocol is generally considered to be determined by the cooling rate used during cryopreservation. Points for consideration are that of warming rate to be applied, avoiding CPA toxicity and osmotic shock (particularly when dealing with vitrified samples where CPA concentrations are typically higher) (Mazur 2004; Muldrew et al. 2004).

#### 1.4.5.1. Warming of slowly cooled cells

For cells that have been cryopreserved using slow cooling rates, arguments can be made and examples given demonstrating advantages of applying both slow and rapid warming protocols or that the warming protocol does not affect cell survival either way (Mazur 2004). For example, for slowly-cooled islets (Rajotte 1999), hepatocytes (Terry et al. 2006; Terry et al. 2010), and blastocysts (Stein et al. 1993), rapid thawing is recommended. Slowly cooled cells may also contain vitrified portions which will benefit from rapid thawing protocols that reduce the chance of devitrification/recrystallisation. However, it has been found that slow cooling through the glass transition temperature (between -150 and -100°C) allows for glass "softening" that prevents occurrence of injurious brittle fractures (Pegg et al. 1997).

However, for some sperm cells, warming rate after slow cooling does not affect post-thaw motility or mobility (Yu et al. 2002). Furthermore, for slowly cooled red blood cells (Miller & Mazur 1976) and arteries (Bujan et al. 2001) slow warming is preferential. The reasons for these discrepancies are likely to be due to differing cell properties, including their ability to tolerate exposure to CPAs at various temperatures. Similarly, where slow cooling has resulted in severe cell shrinkage, lysis upon thawing due to rapidly melting ice and subsequent influx of water into the cell can result in severe cell injury or death.

#### 1.4.5.2. Warming of rapidly cooled cells

For cells that have been rapidly cooled, rapid warming rates are recommended to avoid recrystallisation. During rapid cooling, any ice crystals that form are small as nucleation is spontaneous and crystals do not have sufficient time to grow before growth is restricted by other crystals. However, these crystals are thermodynamically unstable as they have large surface areas relative to volume. In order to reduce free energy, recrystallisation to form larger, more thermodynamically stable crystals is

possible during slow warming protocols. This will likely result in mechanical disruption of plasma membranes and other cell organelles (Mazur 2004). Therefore, for rapidly cooled cells such as sperm (Soler et al. 2003; Yu et al. 2002), rapid warming is recommended.

### 1.4.6. Cryoprotectants (CPAs)

CPAs are included during cryopreservation to protect against the injury mechanisms described above. They can be broadly classed into 2 groups, either penetrating or non-penetrating, and both improve survival during cryopreservation although by different mechanisms.

#### 1.4.6.1. Addition and removal of cryoprotectants

Addition and removal of CPAs must be performed using a protocol that does not exert excessive osmotic stress on the cells (i.e. that the cell does not shrink or swell to a degree that results in cell injury or death). Upon addition of any CPA, cells will initially shrink as water leaves the cell by osmosis in response to the increased solute concentration extracellularly. If cell-penetrating CPA are applied, the cell will swell as CPA crosses the cell membrane. This shrink/swell response occurs as cells possess increased permeability to water than solutes (Meryman 2007) and even before the cell has been cryopreserved can cause lethal damage (Gao et al. 1995). However, if only non-penetrating CPAs are used, this swelling will not be observed and the cell will remain dehydrated.

These osmotic characteristics have been known for a number of years and development of step-wise addition and removal of CPA protocols has resulted. These protocols are designed such that the cell does not shrink or swell beyond its osmotic tolerance and are cell-type and even species specific (Si et al. 2006). Step-wise addition and removal of CPAs have been shown to significantly improve survival of various cell types including oocytes (Isachenko et al. 2004; Wang et al. 2010) and islets (de Freitas et al. 1998). As an additional strategy, some groups have reported improved survival of hepatocytes (Terry et al. 2010) by including glucose in the thawing medium to reduce osmotic stresses.

#### 1.4.6.2. Penetrating CPAs

Penetrating CPAs are small non-ionic molecules that are able to cross the plasma membrane to enter the cell. Commonly used penetrating CPAs include dimethylsulfoxide (DMSO), glycerol and propylene glycol (PG). The efficacy of penetrating CPAs depends on various properties including mode/s of protective action and permeability which are offset by their cytotoxicity.

All penetrating CPAs act by reducing the amount of ice present at any given temperature, thus protecting against solution effects injury resulting from slow cooling profile (Lovelock & Bishop 1959). However, some, notably glycerol, also act to stabilise intracellular macromolecules, protecting against denaturation of proteins that occurs with cooling (Meryman 2007). Membrane permeability is also important as firstly the CPA must be present intracellularly to be effective but also sustained exposure to the CPA at temperatures required for permeation may in itself result in cell death (Mukherjee et al. 2007) and so should be minimised. Consequently, DMSO is often used due to its ready diffusion into cells (Jomha et al. 2009).

#### 1.4.6.3. Non-penetrating CPAs

Conversely, non-penetrating CPAs are large water-soluble molecules that have a different mode of protective action entirely. They typically include raffinose, sucrose and polyethylene glycol. Non-penetrating CPAs protect cells during cryopreservation by promoting cell dehydration by osmosis as their presence outside the cell increases osmolarity. By promoting cell dehydration, the volume of mobile water persisting inside the cells is reduced, thus decreasing the volume of water that could potentially form lethal intracellular ice (Muldrew et al. 2004). Alone, the use of non-penetrating CPAs is not thought to be sufficient to protect cells during cryopreservation but may be used to reduce the required concentration of penetrating CPA. This approach has been utilised for cryopreservation of stem cells where DMSO concentration was able to be decreased when sucrose was used as a CPA (Petrenko et al. 2008).

### 1.4.7. Cryopreservation of hepatocytes

Cryopreservation of hepatocytes would be desirable for a number of applications including xenobiotic metabolism models (Diener et al. 1993; Guillouzo et al. 1999; Li et al. 1999), hepatocyte transplantation (Strain 1994; Terry et al. 2006) and BALs (Magalhaes et al. 2009; Son et al. 2006). Cryopreservation of hepatocytes can be

relatively successful although function and attachment are reduced to varying degrees. Typical protocols employ slow cooling with approximately 10% DMSO (Terry et al. 2006). Other factors affecting success include but are not limited to cell source and method of isolation, freezing cell density, CPA choice, cooling rate, storage temperature and warming rate (Terry et al. 2005; Terry et al. 2010).

### 1.4.8. Cryopreservation of alginate-encapsulated cells

Alginate-encapsulation is thought to protect cells from damage during cryopreservation and this has been demonstrated experimentally for various cell types in various culture formats including islets (Inaba et al. 1996), neurons (Malpique et al. 2010) and hepatocytes (Koizumi et al. 2007; Kusano et al. 2008; Mahler et al. 2003). Suggested mechanisms of protection include increased time for cell dehydration as ice initially forms outside the alginate, provision of a support scaffold, and a lessened apoptotic response (Kusano et al. 2008; Mahler et al. 2003).

### 1.4.9. Cryopreservation of interconnecting cells

Cryopreservation of interconnecting cells is more complex than cryopreservation of single cell suspensions and recoveries are typically worse. This is due to a number of compounding factors such as incomplete CPA permeation, problems of intra- and intercellular ice and heat transfer.

#### 1.4.9.1. CPA permeation

For successful cryopreservation, including vitrification, CPAs must permeate the tissue. Protocols for ensuring sufficient CPA permeation are tissue/cell-type specific and equilibration of the tissue with CPA may require a few hours (corneas (Wusteman et al. 2004) but may be more rapid (arteries and ovarian tissue (Newton et al. 1998; Wusteman et al. 2004)). Temperature at which equilibration is attempted is important and higher temperatures result in more rapid perfusion but are also associated with increased cytotoxicity. A common problem is that whilst cells located at the tissue surface contain sufficient CPA, cells located deep in the tissue do not and although continued perfusion will rectify this, cytotoxicity at the surface of the tissue may result (Karlsson et al. 1993).

#### 1.4.9.2. Intracellular ice formation

As described previously, IIF is often lethal but strategies exist to reduce or avoid it, particularly for cryopreservation of single cell suspensions. Although these strategies may also be applied during tissue cryopreservation, IIF in tissues or interacting cell clusters (such as mammalian embryos) remains more likely. Water transport characteristics differ significantly between single cells and multicellular components as water must exit cells sequentially from centre to periphery (Ehrhart et al. 2009). This makes dehydration harder to achieve thus increasing likelihood of IIF (Balasubramanian et al. 2006).

This mechanism of injury is further compounded in cells connected by gap junctions (in either monolayer or 3D culture formats) as ice may propagate between cells via these cell-cell contacts. This mechanism of injury has been demonstrated in a variety of interconnecting cell types including salivary gland cells (Berger & Uhrik 1996) and kidney cells (Acker et al. 2001). A comparison study was also performed in kidney cells that do not form cell-cell contacts and post-thaw recoveries were much improved (although no direct comparison was made between the 2 kidney cell types) (Armitage & Juss 2003).

#### 1.4.9.3. Intercellular ice formation

It has been suggested that intercellular ice formation can damage cell suspensions in high densities or cells comprising multicellular components by mechanical force as individual cells are forced into small spaces between ice crystals which can result in cell rupture if cells are sufficiently deformed (Muldrew et al. 2004; Takamatsu & Rubinsky 1999).

In addition, intercellular ice formation is more problematic in tissues as it also has the capability to disrupt tissue structure that is required for maintenance of function (Liu & Mcgrath 2005; Pegg 2006). Another mechanism by which intercellular ice can damage tissues during cryopreservation is the formation of ice lenses. Ice lenses form deep within tissue when supercooled liquid nucleates. Water is then drawn to the ice via capillary action through channels dehydrating cells in the locality. Upon thawing, the cells then lyse when ice melts and water rushes back into these desiccated cells (Muldrew et al. 2000).

#### 1.4.9.4. Heat transfer

Heat transfer is also an important issue during cooling and warming in larger volumes. Heat gradients may exist within samples, meaning that the desired cooling rate is not applied evenly across the entire tissue (i.e. cells centrally-located will be cooled slower than those peripherally-located). Obviously the degree to which this occurs depends upon the mass of the tissue to be cryopreserved and is not necessarily detrimental to cell survival if the range of temperatures applied falls within the acceptable range of cooling rates for cell survival. Similarly, during warming centrally-located cells will be slower to warm, making devitrification and/or ice recrystallisation more likely. At the same time, thawed cells peripherally-located may be exposed to high concentrations of injurious CPAs over longer time periods (Karlsson & Toner 1996).

### 1.4.10. Assessment of success following cryopreservation

Numerous methods of assessing success following cryopreservation exist and "viability" is often stated as an indicator of good recovery. However "viability" remains undefined scientifically, but the term is often used to describe membrane integrity, assessed using dyes such as trypan blue and propidium iodide (PI). Whilst these techniques are valuable for first-line assessment of cell integrity, they are relatively simplistic and typically overestimate success. More complex assays, such as cell attachment in culture or specific metabolic assays and signalling, offer more realistic estimations of cell survival as they require cell surface recognition etc.

However, what should be used as a means of assessing cryopreservation success should be directly linked to the intended end-application of the cells. For example, for cryopreserved skin grafts and cartilage, maintenance of structure is important whereas the survival of the cellular population is perhaps less vital. Conversely, for regenerative medicine applications such as islet transplantation, the aim is to deliver sufficient functioning cells: that is, the largest possible number of cells with the highest possible glucose stimulation index response (i.e. similar to that of unfrozen islets).

Similarly, for a BAL, the aim is to deliver a biomass capable of replacing liver function: (metabolism, synthesis and detoxification), without posing additional risk to the patient. Therefore, indicators of success following cryopreservation should be:

Viability: should be maintained. Membrane integrity can provide a means of quantifying initial recovery in post-thaw cultures. It can be assessed rapidly and over

time to ensure that membrane integrity is retained following cryopreservation. Whilst this assay does not offer a holistic assessment of the biomass, it serves as a useful indicator of success following cryopreservation. Maintenance of membrane integrity also suggests that cells remain intact and that debris is not released into post-thaw cultures, which may cause further problems as described above.

Cell number: large cell numbers are estimated to be required, as described earlier. By assessing cell number in post-thaw cultures, whether or not this has been achieved can be assessed. Additionally, cell growth can be assessed which may provide further confidence in the integrity of the biomass. Finally, maintenance of cell number may indicate that cell debris and DNA is not "unaccounted" for (i.e. there is less risk of it returning to the patient on release from dead cells where it could potentially go on to pose health risks).

Function: complete expression of function and repertoire of the cells is the gold standard. Ideally, this would include all liver functions including metabolic, synthetic and detoxification processes. These could be assessed using various methods such as assays to assess cytochrome P450 function and measurement of synthesis and secretion of hepato-specific proteins released into conditioned medium during post-thaw culture.

However, the exact methods chosen for assessing success following cryopreservation should be specific to the cells and their culture format.

Cell number can be assessed readily for cells in suspension using a haemocytometer. However, for ELS cultures, cells must first be released from the alginate matrix. This can be achieved by using EDTA to chelate divalent cations (calcium in this case) used to crosslink alginate chains. Once depolymerised, cell spheroids can be separated from alginate via centrifugation. Cell spheroids can be disaggregated using pipetting and vortexing before cells are counted.

Again, viability (membrane integrity) can be readily assessed for cells in suspension using trypan blue exclusion or similar. However this technique cannot be applied to encapsulated cells as counting individual cells is not possible in this 3D format. Although cells could be released as described above, this would likely result in a possible loss of viability due to shear stresses applied during disaggregation of cell spheroids. However, viability may also be assessed using fluorescent staining, where dye concentrations relate to overall functional biomass. Fluorescent stains may be used to stain ELS within alginate and do not therefore require disruption of the culture format

(advantageous cf. trypan blue or similar). Staining of the ELS can be visualised using microscopy and images captured using a camera. Viability may then be objectively quantified using image analysis to compare relative densities of the 2 fluorescent stains.
Functions of ELS can also be measured. As described earlier, the liver performs a number of wide-ranging functions. However, as the cellular component of the LG BAL consists of HepG2 cells, it is not appropriate to measure all of these functions throughout this thesis. HepG2s possess only limited cytochrome P450 function, which although inducible, remains low in fresh cultures, and even lower in frozen/thawed cultures, making this a difficult and expensive function to measure. However, HepG2 cells do possess good protein synthetic functions and are capable of bilirubin conjugation. Bilirubin conjugation could not be measured in-house and was therefore an expensive assay and requires addition of potentially cytotoxic bilirubin. However, synthetic function can be measured simply and reliably using enzyme-linked immunosorbent assay (ELISA). Furthermore, ELISA does not require addition of components to culture medium, merely collection of conditioned medium. The two former assays have been used strategically, whilst ELISAs for synthesised and secreted proteins were used in all experiments.

## 1.5. Aim and Hypothesis

The aim of the following work was to develop a preservation protocol such that encapsulated liver cell spheroids can be delivered with high functional recovery as and when required for use within a bioartificial liver for treatment of acute liver failure.
Whilst much is known about cryopreservation of single cells, including hepatocytes, less is known about cryopreservation of inter-connecting cell types or encapsulated cells. However, cryopreservation of ELS is required for a BAL to be a clinically-relevant treatment option.

**Hypothesis: By studying cryopreservation-related injuries to ELS using protocols suitable for single cells, mechanisms of injury in ELS can be identified. Applications of specific biophysical and molecular processes can then be applied to improve ELS recoveries to levels suitable for BAL applications.**

Overall, the aims of this thesis were to:

Characterise cell death and injury mechanisms during cryopreservation

As ELS are intended for use within a BAL for emergency use, cell death and the time scale over which it occurs must be characterised in order to ensure that ELS are

deliverable within a suitable time frame. Furthermore, the injury mechanisms resulting in cell death will be identified. Much is known about the potential damaging processes that occur during cryopreservation such as intracellular ice formation as a result of too rapid cooling rates and this will be investigated.

Develop strategies to limit or prevent these injury mechanisms

Once these injury mechanisms have been identified, steps will be taken to reduce their damaging action. Strategies will be developed with the specific intention of reducing individual injurious mechanisms. By tackling each injurious mechanism, it is hoped that the effect will be cumulative to result in an improved cryopreservation protocol producing ELS that are sufficiently functional within a sufficiently rapid time frame to treat ALF.

Transfer this knowledge from small scale static culture to those appropriate for ELS cultured in dynamic format in a bioreactor for a BAL

Steps 1 and 2 will be investigated using a culture system that will model the ELS that would be eventually used within in a BAL due to both practical and financial reasons. In the model system, cell culture will be performed within a static system (resulting in lower cell densities within alginate beads) and in smaller volumes. However, once the optimal protocol has been devised, this will be transferred to a larger scale model, more similar to that which would be used in reality.

Test this protocol for another potential cell source for a BAL, namely primary human hepatocytes

Finally, the developed cryopreservation protocol will be tested in primary human hepatocytes (PHH). PHH represent a potential alternative cell source for a BAL, although availability is likely to be an issue. However, by testing the protocol in a similar cell type, transferability of the protocol may be trialled.

# CHAPTER 2

# General Methods

This chapter describes the general methods used throughout this thesis. These methods were primarily applied to 3D cultures of alginate-encapsulated liver cell spheroids but also to monolayer cultures of adhered cells. Cell culture reagents were obtained from Invitrogen. All other reagents were obtained from Sigma Aldrich unless otherwise stated.

## 2.1. HepG2 cell line culture

### 2.1.1. Culture medium preparation

2.1.1.1. HepG2 culture medium (complete culture medium)

Materials

α-MEM with ribonucleosides and deoxyribonucleosides (Invitrogen 32571)

10% Foetal Calf Serum (FCS) (Hyclone CH30160-03)

100U/ml Penicillin/0.1mg/ml Streptomycin (Lonza DE17-603E)

1.25μg/ml Fungizone (Gibco 15290-026)

50μg/ml Linoleic Acid Bovine Serum Albumin (BSA)

0.04μg/ml Hydrocortisone

0.04μg/ml Thyroid Releasing Hormone (TRH)

9.5μg/ml Insulin (Actrapid 8-0201-01-203-3)

0.002μg/ml Sodium Selenite

Method

For media preparation, additives were pooled in a 50ml centrifuge tube and filtered through a 0.2μm filter prior to addition to culture media.

2.1.1.2. Culture medium for HepG2 cells encapsulated in alginate using the Inotech-syringe pump system (high glucose medium)

Materials

Complete culture medium

45% D-Glucose solution (Sigma G8769)

Method

D-glucose solution was added to complete culture medium to achieve a final concentration of 25mM (4.44ml/500ml bottle).

2.1.1.3. Culture medium for HepG2 cells encapsulated in alginate using the JetCutter system (FFP medium)

Materials

Serum-free high glucose culture medium (25mM glucose)

Fresh frozen plasma

1M calcium chloride

Heparin (Multiparin, CP Pharmaceuticals)

Method

*2.1.1.3.1. Preparation of fresh frozen plasma*

Human fresh frozen plasma (FFP) was obtained, thawed and pooled from multiple donors by blood group. During initial collection of FFP, sodium citrate was added as an anticoagulant. Sodium citrate cannot be used with the LG BAL as it is a calcium chelator and its use would result in depolymerisation of alginate. Heparin (40IU/ml) was added as an alternative anticoagulant. To neutralise the citrate, 4µl/ml 1M calcium chloride was added to prevent depolymerisation. 50ml aliquots were prepared and stored at -20°C until required. Prior to use FPP was thawed, centrifuged at 1000g for 20 minutes and supernatant decanted.

*2.1.1.3.2. Preparation of FFP media*

56ml FFP was added to serum-free high glucose culture medium to achieve a final concentration of 10% FFP.

**2.1.2. Culture of HepG2 cell line as monolayer cultures of adherent cells**

HepG2 cells were originally obtained from the European Collection of Cell Cultures (ECACC), and adapted to long term culture in complete culture medium as described in section 2.1.1.1 above and used by the Liver Group (LG) for a number of years. These cells were passaged once per week.

Materials

HBSS (without calcium and magnesium)

Liver Group (LG) trypsin (0.25g/L Trypsin 0.1g/L EDTA (Invitrogen # 15400-054), 1g/L glucose in citrate saline (4.4g/L trisodium citrate.2$H_2O$, 10g/L KCl))

Complete culture medium (as described in 2.1.1.1)

LG freezing mix (FCS containing 10% DMSO)

2% Trypan blue in PBS

Haemocytometer

Tissue culture flasks (Nunclon surface, Nunc)

2ml cryovials (Nunc)

Polystyrene container lined with cotton wool

37°C water bath

Method

Cell culture work was performed in a class II microbiological safety cabinet using disposable sterile plastic ware. HepG2 cells are adherent on tissue culture plastics and were cultured at 37°C within a humidified atmosphere with 95% air, 5% CO2 in an incubator. Medium was changed every 2-3 days and cells were passaged once per week by trypsinisation when 70-80% confluent.

To passage cells, medium was removed and cells were washed three times with HBSS. 1.5ml LG trypsin was added per 25cm$^2$ growth area and incubated for no more than 6 minutes at 37°C. After this time, cells were detached from the surface by knocking the side of the flask firmly. Double the volume of warmed complete medium was added to inactivate the trypsin and cells were centrifuged at 300g (1200rpm) at room temperature for 4 minutes. Supernatant was discarded and cells were resuspended in complete culture medium. Cell clumps were disaggregated using a 21G needle no more than 3 times. Cells were reseeded as required.

2.1.2.1. Determination of cell number and viability

Method

Cell number and viability was determined using trypan blue exclusion of non-viable cells. 160µl of HBSS, 20µl of trypan blue and 20µl of homogeneous cell suspension was mixed and incubated for 2 minutes. 9µl of this solution was loaded onto a haemocytometer chamber and both viable and non-viable cells were counted.

2.1.2.2. Cryopreservation and revival of HepG2 cell line

Cell lines were maintained in culture for no more than 10 continuous passages. Therefore, cell bank stocks of early passage HepG2 cells were prepared.

Method

*2.1.2.2.1. Cryopreservation for maintaining HepG2 cell stocks for monolayer cultures*

Following trypsinisation, cells were resuspended in complete culture medium at a density of either $2x10^6$cells/ml or $2x10^7$cells/ml. An equal volume of LG freezing mix was added and 1ml aliquots transferred to cryovials. Cryovials were placed in a polystyrene container lined with cotton wool which was left at -80°C overnight, producing slow-cooling conditions. Cells were then transferred to the vapour phase of liquid nitrogen for long term storage.

*2.1.2.2.2. Cell revival*

To revive cells, cryovials were removed from the vapour phase of liquid nitrogen and thawed quickly using a 37°C water bath until the last ice crystal had melted. Thawed cells were transferred to a 50ml centrifuge tube and placed on ice. Complete culture medium was added such that the volume was doubled over 5 minutes, then again over the next 5 minutes and once more over the next 5 minutes. The cells were centrifuged at 300g (1200rpm) for 4 minutes at room temperature. The supernatant was discarded and cells resuspended in complete culture medium and plated as required. Following overnight attachment, cells were washed with HBSS to remove any unattached cells and warmed complete culture medium replenished. This yielded cell recoveries of approximately 50% in terms of attachment although this was not measured routinely.

## 2.2. Alginate encapsulation and 3D culture

### 2.2.1. Encapsulation of HepG2 Cells using the Inotech Syringe Pump System

Inotech encapsulation was used when only small volumes of beads were required for experiments. The Inotech IER-20 Cell Encapsulator system (Inotech, Dottikon, Switzerland) works on the principle of interruption of a laminar liquid jet by vibration to form equal sized droplets. An electrostatic dispersion unit adds charge to each droplet so that each droplet repels each other so that they do not collide in flight or on the surface of the polymerisation solution (Figure 2-1 overleaf). Alginate beads are polymerised using divalent cations (most commonly calcium) to crosslink alginate chains.

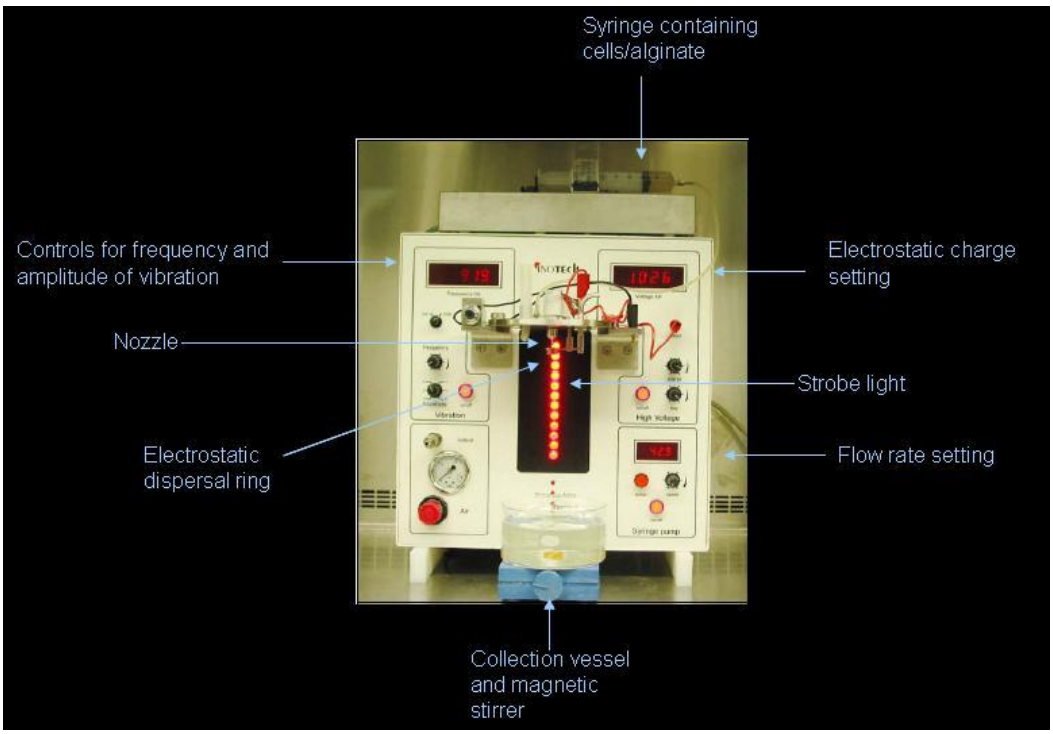


**Figure 2-1. Inotech IER-20 Cell Encapsulator System.**

## Materials

<u>100ml 2% Alginate – pH 7.4</u>

0.15M Sodium chloride

1M Sodium hydroxide

15mM HEPES buffer

2% w/v Alginic acid sodium salt from *Macrocystispyrifera* kelp (Sigma A2033)

<u>Polymerisation Buffer – pH 7.4</u>

0.15M Sodium chloride

1M Sodium hydroxide

15mM HEPES buffer

0.204M Calcium chloride

0.05 % w/v Pluronic acid (Sigma P-1300)

Volumetric flasks

Schott bottle

Magnetic flea

Syringe filter (autoclaved)

Baked Pyrex glass beakers (3 hours at 180°C)

Stainless steel forceps (autoclaved)

Inotech syringe pump system (Inotech, Dottiken, Switzerland)

Bottomless beaker (autoclaved)

Elastic band (autoclaved)

Nylon mesh (ethanol sterilized)

DMEM

High glucose (HG) medium

Method

2.2.1.1. Preparation of 2% alginate solution

100ml of HEPES buffered saline solution was prepared and pH adjusted to 7.4 using 1M NaOH. This solution was put into a foil-covered 250ml Schott bottle with a magnetic stirrer. Alginic acid was added slowly and the solution was stirred slowly overnight. The next day, the alginate solution was autoclaved for 10 minutes at 121°C and allowed to cool to room temperature before use.

2.2.1.2. Preparation of polymerisation buffer

A 0.204M $CaCl_2$ solution was prepared in HEPES buffered saline and pH adjusted to 7.4 using 1M NaOH. Before, use polymerisation buffer was autoclaved at 121°C for 25 minutes and allowed to cool to room temperature. Pluronic acid (0.2g) was dissolved in polymerisation buffer and filter sterilised immediately before use.

2.2.1.3. Preparation of alginate cell suspension

Adherent HepG2 monolayer cell cultures were trypsinised and the pellet resuspended in 5ml of HG medium and cell number and viability determined by trypan blue exclusion. Cells were diluted to a final concentration of $1x10^6$/ml in high glucose culture medium which was then diluted one to one with 2% alginate solution to achieve a final cell concentration of $0.5x10^6$/ml in 1% alginate. This solution was drawn up through a 50µm filter into a 50ml syringe and attached to the syringe pump of the Inotech.

2.2.1.4. Cell encapsulation and bead collection

The cell/alginate solution was passed through the encapsulator at an arbitrary flow setting of 12.7 through a 200µm nozzle. Vibration was set to 1295Hz (vibration amplitude 3). This yielded spherical beads of approximately 500µm in diameter. Electrostatic charge was set to 0.5kV. The beads fell into the polymerisation buffer where they were left to polymerise for 10 minutes. After this time, beads were separated from the polymerisation buffer by pouring them into a beaker containing a 200µm nylon mesh bottom. The beads were washed twice with DMEM to remove any remaining polymerisation buffer. Beads were transferred to 15ml centrifuge tubes and resuspended in complete high glucose media. Once settled, the volume collected was estimated and beads were diluted with complete high glucose media.

2.2.1.5. Static culture of Inotech encapsulated HepG2 cells

Method

Beads were resuspended in a 175cm$^2$ tissue culture flask with complete high glucose media at a ratio of 1:32 beads:medium. 8.25ml of this mixture was transferred into each well of a 6 well plate containing a 100µm cell strainer. The inclusion of a cell strainer allowed beads to be easily removed during media changes or assays and allowed for diffusion of media from both top and bottom. Media was replenished every 2-3 days unless otherwise stated.

### 2.2.2. JetCutter cell encapsulation

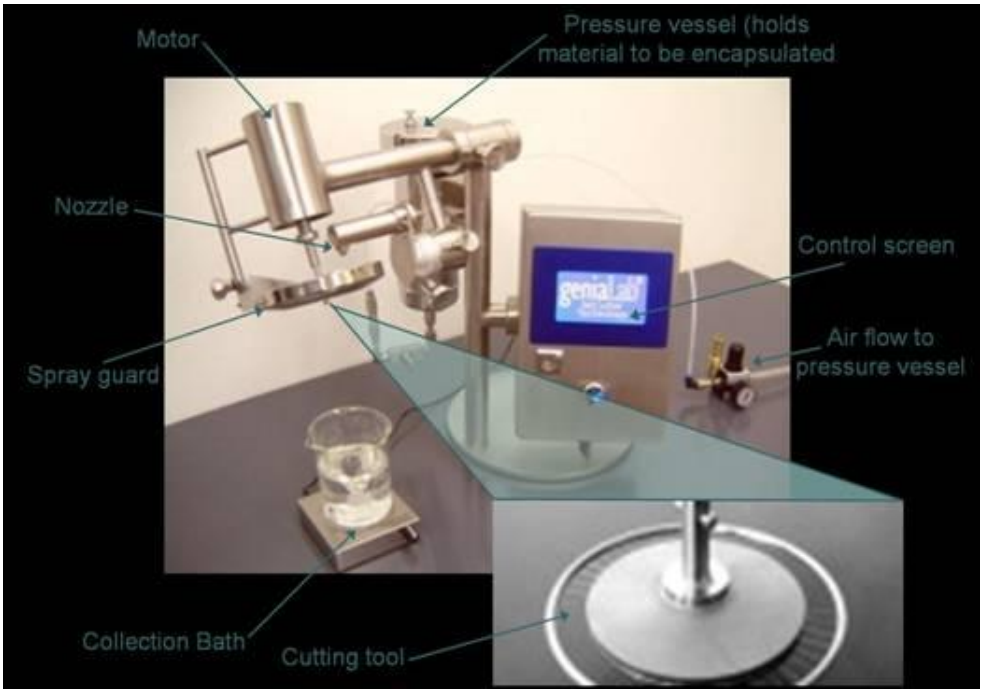


**Figure 2-2. Jetcutter encapsulation system.**

When larger volumes (greater than ~30ml beads) were required, the JetCutter encapsulation system was used. The JetCutter system (geniaLab®) also works on the principle of laminar flow interruption which is achieved in this system using a cutting tool with asymmetrically arranged wires which results in cylindrical droplets that form spheres due to surface tension (Figure 2-2). As with the Inotech system, polymerisation of alginate is achieved using divalent cations.

Materials

2% alginate solution

0.17M Polymerisation buffer

HG Culture Media

DMEM medium supplemented with 10% FCS, P/S and Fungizone

Baked Pyrex beakers (3 hours at 180°C)

JetCutter components (pressure vessel, inline filter, nozzle holder and nozzle assembled and autoclaved, cutting tool, cutting disc, collection cover) (autoclaved)

5l stainless steel basin (autoclaved)

Plastic beaker for collecting waste from JetCutter

Plastic bottomless beaker and elastic band (autoclaved)

200µm nylon mesh (ethanol sterilised)

Magnetic Stirrers (autoclaved)

Stainless steel spatula (autoclaved)

Sterile consumables including 15ml and 50ml centrifuge tubes, 50ml syringes, 25ml and 50ml serological pipettes, Gilson tips and T175 culture flasks

Method

2.2.2.1. JetCutter setup

Cells were diluted in a mixture of one part 2% alginate to one part HG culture medium to a final concentration of between $1.5x10^6$/ml and $2x10^6$/ml. This mixture was poured into the pressure vessel and immediately capped and stirring motor set to 50rpm. Air pressure was applied and the pressure outlet tap opened to allow flow of alginate. Flow was calibrated to 0.33ml/sec by adjusting air pressure. Run parameters were set as follows:

Flow rate: 0.33ml/sec

Nozzle size: 350µm

Wires: 60

Wire diameter: 100

Motor speed: 3600rpm

JetCutter software was used to calculate optimal angle of inclination of the cutting tool which was then attached along with the cutting disk and collection cover. A 5L basin containing 3L of polymerisation buffer was placed on a magnetic stirrer under the nozzle and set to stir at a low speed.

2.2.2.2. Cell encapsulation and bead collection

Once setup was completed, the outlet tap on the pressure vessel was opened to commence liquid flow and the JetCutter motor started. Initial flow through was collected in a beaker and discarded. Once a clean flow through was established, beads were collected in the basin containing polymerisation buffer. Beads were then poured from the basin into a 1L beaker and removed from the polymerisation buffer using a beaker with a 200µm mesh bottom. Beads were washed with DMEM and transferred to 50ml centrifuge tubes where they were resuspended in either complete high glucose medium or complete high glucose medium containing 10% FFP. Once settled, the volume of beads was estimated and diluted further with medium as required.

2.2.2.3. Culture of JetCutter encapsulated HepG2 cells

*2.2.2.3.1. Fluidised bed bioreactor*

The fluidised bed bioreactor (FBB) setup was used when high cell densities were required and was normally performed by other colleagues. The FBB setup allows for greater mass transfer and provides greater control of cell culture environment. Oxygen supply, pH and temperature are controlled using a fermentor and individual amino acid supplementation may be performed as required. Beads are cultured in a separate cylindrical chamber and fluidised by medium flowing from the fermentor via a reservoir into the base of the chamber. Beads move in a circuit from the base of the chamber to the top of the chamber before falling to the base again due to gravitational forces (Figure 2-3).

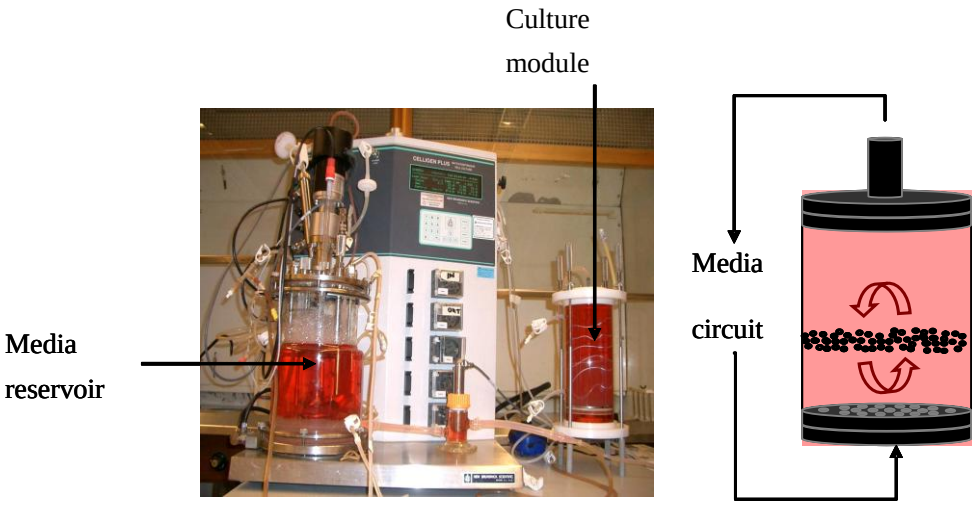


**Figure 2-3. FBB culture system setup.**

Materials

Celligen Plus Bioreactor (sterilised)

pH probe

pH 4 and 7 standards

External pH meter

Dissolved oxygen (DO) probe

FFP medium

Nitrogen gas cylinder

Oxygen gas cylinder

100mM amino acids

Fish pump

Silicon tubing

Peristaltic pump

5 and 20ml syringes

21G needles

Biocommand Plus Software

10L glass bottles (autoclaved)

Method

**5.1.1.1.1.1 Preparation for bioreactor setup**

This work was performed by other colleagues. The bioreactor was prepared according to the manufacturer's instructions. Briefly, prior to beginning bead culture, the pH probe was calibrated using pH standards, bioreactor assembled and sterilised by autoclaving. The sterilised vessel was filled with FFP medium. DO and pH probes were inserted and left to polarise overnight. The pH probe was recalibrated against an external pH meter. DO probe was calibrated using nitrogen gas (0%) and oxygen gas (100%).

**5.1.1.1.1.2 FBB culture**

Biocommand Plus software was used to monitor, control and record output throughout each FBB experiment. Bead volume following JetCutter encapsulation was quantified and no more than 800ml beads were cultured within the FBB at any time. Beads were transferred to the chamber and fluidised so that a 2-fold increase in bed height was achieved. Media changes were performed by replacing spent medium in the reservoir and were performed as required but normally on days 5, 8, 10 and 11 and a ratio of 1:58

bead:medium was maintained throughout the experiment. In addition, amino acid supplementation was performed according to results from a mathematical model of amino acid depletion throughout the experiment.

2.2.2.4. Rotating cell culture system

The rotating cell culture system (RCCS) was used when high cell densities were required for experiments or when beads had previously been cultured using the FBB setup. The RCCS consists of a cylindrical culture module with a gas-permeable central core (silicon membrane). The module is rotated such that the beads are held in constant freefall. Therefore, the RCCS provides a similar culture environment to the FBB (Figure 2-4) for small volumes of ELS.

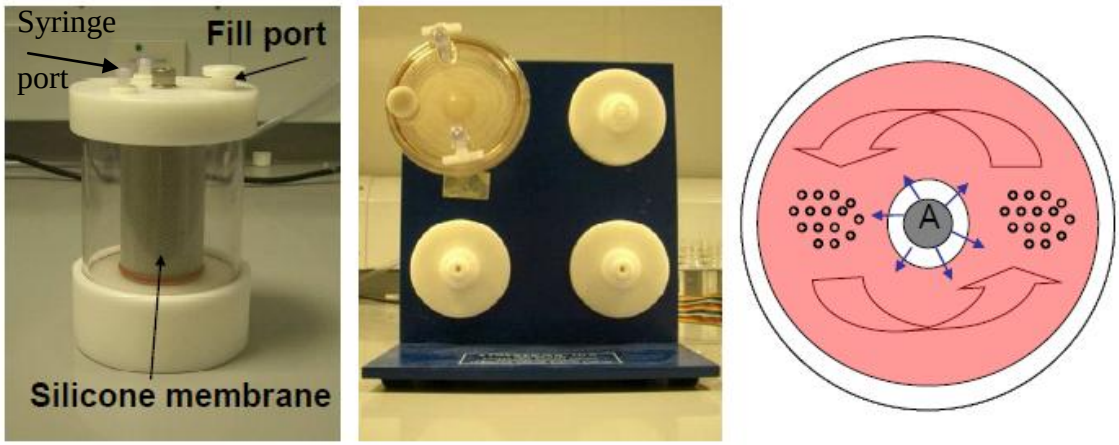


**Figure 2-4. RCCS setup.**

**Cylindrical culture module (left). Culture module attached to RCCS base (middle). Schematic demonstrating oxygen flow and beads held in continuous freefall (right).**

Materials

FFP medium

25ml serological pipette

Culture module (autoclaved)

RCCS base

20ml syringe

Motor

Method

Beads were suspended in a small volume of medium and transferred to the culture module using a pipette via the fill port. The remainder of the module was filled with

medium, again via the fill port which was then closed. An empty syringe and a media-filled syringe were attached to the module at the 2 syringe ports. Air bubbles were evacuated from the module by tilting the module so that the bubble could be removed using the empty syringe. This volume was then replaced using media from the second syringe. The module was then attached to the RCCS base and rotated at 10rpm within a 37°C humidified incubator. Bead:medium ratio was maintained at 1:50 throughout culture. Media was changed every 24 hours by removing the module from the base and standing the module on its end. Once beads had settled, spent media was removed via the fill port and replaced with fresh media.

## 2.3. Evaluation of cell number from 3D cultures

### 2.3.1. Removing cells from alginate

EDTA was used to chelate calcium ions from the alginate used to encapsulate the 3D cell spheroids which subsequently dissolved, releasing the HepG2 cell spheroids.

Materials

HBSS

16mM EDTA in 0.15M NaCl (pH 7.4)

1x sterile PBS

6-well plates

Small spatula

2ml microfuge tubes

15ml centrifuge tubes

Vortex

2.3.1.1. Method for beads maintained in 6-well plates

Beads were removed from culture in their cell strainers and washed in 8ml HBSS in a new 6-well plate. Using a small spatula, the beads were scraped from the cell strainers into 2ml microfuge tubes. 1.6ml EDTA was added and beads were incubated for 10 minutes at 37°C until dissolved. Beads were centrifuged at 13200g for 5 minutes to pellet spheroids. The supernatant was discarded and pellet of multicellular spheroids vortexed before being resuspended in an appropriate volume of PBS according to day following encapsulation, as below:

Day 0 and 1 – 200µl

Day 2 and 3 – 300µl

Day 4 and 5 – 500µl

Day 6 onwards – 1ml

The cell spheroids were disaggregated using further vortexing and pipetting to yield a single cell suspension.

2.3.1.2. Method for beads maintained in FBB or RCCS

Beads were aliquotted into 15ml centrifuge tubes in 1ml volumes and 14ml EDTA added. Beads were incubated at 37°C for 10 minutes until dissolved and then centrifuged at 4000g for 10 minutes. The supernatant was discarded and cell pellet vortexed before being resuspended in 5ml PBS. The spheroids were disaggregated using further vortexing and pipetting.

### 2.3.2. Quantification of cell number using the Nucleocounter System

Cell numbers were quantified using the Nucleocounter System, an integrated fluorescence microscope. Cell samples are lysed using acid before pH is neutralised and samples loaded into Nucleocassettes. Nucleocassettes contain propidium iodide (PI) within their flow channels which stains lysed cell nuclei. A fluorescent image of a 2µl sample volume is captured allowing cell concentrations (within range of $5x10^3$ - $2x10^6$/ml) to be determined.

Materials

Reagent A100 – lysis buffer (Chemometec 910-0003)

Reagent B – stabilising buffer (Chemometec 910-0002)

2ml microfuge tubes

Vortex

Nucleocassettes (Chemometec 941-0002)

Nucleocounter

Method

500µl cell suspension (or less as appropriate at earlier stages of 3D culture) was transferred to a new 2ml microfuge tube. An equal volume of Reagent A100 was added and vortexed for 10 seconds. An equal volume of Reagent B was added and vortexed for a further 10 seconds. The samples were loaded into individual Nucleocassettes which were loaded into the Nucleocounter where cell concentration was determined.

Cell number/ml alginate was calculated as below, by applying a multiplication factor (MF):

$$MF = 3 \times V_B^{-1} \times V_{PBS}$$

where $V_B$ = volume of alginate beads (ml)

and $V_{PBS}$ = volume of PBS used to resuspend pellet (ml)

Total cell number was calculated as below, again by applying a MF.

$$MF = 3 \times V_{PBS}$$

where $V_{PBS}$ = volume of PBS used to resuspend pellet (ml)

## 2.4. Calculation of viable cell number

Viable cell numbers at each time point were calculated as the cell number per ml alginate multiplied by the average viability at that time point. Average viability was determined using fluorescent staining with image analysis, described fully in the following section.

## 2.5. Evaluation of cell viability and basal metabolic activities

### 2.5.1. Qualitative assessment in beads – Fluorescein diacetate/propidium iodide staining

Fluorescein diacetate (FDA) is cell membrane-permeable and when hydrolysed by esterases present in metabolically active cell cytoplasm, emits a green fluorescence. PI is cell membrane-impermeable and emits a red fluorescence when bound to DNA. Therefore, PI staining will occur only when cell membranes are compromised, representing non-viable cells, whilst FDA staining diminishes in dying cells.

Materials

1mg/ml FDA (Sigma F7378-5G) in DMSO

1mg/ml PI (Sigma 70335-5ML-F) in water

1x PBS containing calcium and magnesium

Microscope slides

Tissue

Coverslips

Nikon Eclipse microscope

DX1200 camera

FDA filter block (excitation filter of 465-495nm, emission filter of 515-555nm)

PI filter block (excitation filter of 510-560nm, emission filter of 590nm)

Method

Approximately 250µl alginate beads were transferred to a 1.5ml microfuge tube, allowed to settle and medium aspirated. Beads were washed with 1ml PBS, then 500µl PBS and resuspended in 500µl of PBS. 20µl PI and 10µl FDA were added, gently mixed and incubated for 90 seconds. PBS was aspirated and beads washed a further 2 times as before. Beads were resuspended in 500µl of PBS and transferred to a microscope slide. Excess liquid was removed using tissue if required and a coverslip was placed over the beads. The beads were then visualised using a fluorescence microscope. Phase contrast, live and dead images were captured using a DX1200 camera and Lucia imaging software. FDA and PI images were captured at x4 magnification at exposures of 64 and 250ms respectively.

### 2.5.2. Quantitative assessment in beads – image analysis

Cell viability was quantified from captured images using image analysis. Macros were written to determine the density of each stain within a given field.

Materials

Fluorescein Diacetate Macro

```
ClearBinary();
WaitText(3,"Click on areas omitted by macro to add them, then right click and click OK");
DefineThreshold(0,106,14,13,255,216,7);
_DefineThreshold();
Threshold();
MeasureField();
_FieldData();
```

Propidium Iodide Macro

```
ClearBinary();
WaitText(3,"Click on areas omitted by macro to add them, then right click and click OK");
DefineThreshold(106,8,6,255,61,18,7);
_DefineThreshold();
Threshold();
MeasureField();
```

_FieldData();

Method

Viability was calculated as below and expressed as the percentage of live cells within the total field.

$$viability\,(\%) = \frac{FDAdensity}{FDAdensity + PIdensity} x100$$

**2.5.3. Quantitative assessment – tetrazolium salt reduction assay**

Tetrazolium salts are reduced to a formazan product by mitochondrial and microsomal reductases in metabolically active cells. The amount of product can be directly related to these active cells and can be quantified using absorbance readings. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was used here.

Materials

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma M5655)

Sterile PBS

4mM HCl in isopropanol

16mM EDTA in 0.15M NaCl

Small spatula

21G needle

2ml microfuge tubes

Plate sealer

Orbital plate shaker

Spectrophotometer at 570nm

Method

0.75mg/ml stocks of MTT were prepared in sterile PBS and stored in single-use aliquots at -20°C. 250µl beads were washed twice with sterile PBS and transferred to 2ml microfuge tubes (with pierced lid) using spatula. 1ml pre-warmed MTT was added to each tube. Cells were incubated at 37°C in a humidified incubator for 3 hours until blue/purple crystals had formed. MTT was aspirated and beads dissolved and cells pelleted as described in section 2.3.1 above. The pellet was washed twice using sterile PBS. 350µl acidified isopropanol was added to each tube and tubes were shaken using orbital plate reader for 30 minutes until all crystals had dissolved. Cells were then microfuged at 13200g for 5 minutes, and supernatant transferred to a 96-well plate and absorbance at 570nm measured.

## 2.6. Quantification of total protein content using bicinchoninic acid protein assay

The bicinchoninic acid (BCA) assay was used to determine protein content of samples. A colour change from green to purple indicated presence of protein which could be quantified using spectrophotometry and comparison to a standard curve of known protein content.

Materials

PBS

Lysis buffer: 0.1M NaOH, 0.05% Triton X in PBS

BCA Reagent A: 1% BCA, 2%$Na_2CO_3$, 0.16% NaK tartrate, 0.4% NaOH, 0.95% $NaHCO_3$ (pH 11.25)

BCA Reagent B: 4% copper sulphate

2mg/ml bovine serum albumin (BSA) (Sigma, P0834-10X-1ML)

Lysis buffer: 5% sodium dodecyl sulphate in 0.1M NaOH

Plate sealer

Orbital plate shaker

96 well clear flat bottomed culture plate

Spectrophotometer at 570nm

Method

Cells were washed twice with PBS prior to assay and lysed using an appropriate volume of lysis buffer. A standard curve was prepared using BSA standard by serial dilution in lysis buffer (1-0.03mg/ml). Immediately prior to the assay, BCA reagents A and B were mixed at a ratio of 50:1 to form working solution. 20µl standard or sample was added to each well of a 96 well plate along with 100µl working solution. The plate was sealed, shaken for 30 seconds using an orbital plate shaker and incubated at 37°C for 30 minutes. Absorbance was measured using a spectrophotometer at 570nm.

## 2.7. Quantification of hepato-specific proteins synthesised and secreted in culture

An enzyme-linked immunosorbent assay (ELISA) was used to quantify hepato-specific protein synthesis and secretion. Antigen is captured by capture antibody (Ab) and detected and quantified using a second detection Ab. Typical detection systems include alkaline phosphatase and horseradish peroxidise (HRP), as here. The substrate for HRP is hydrogen peroxide which is cleaved and coupled to the oxidation of

o-phenylenediamine (OPD) to form an orange product that can be quantified using a spectrophotometer.

A standard curve was run on each plate. To ensure that test samples fell within range of this standard curve, samples were diluted serially with an appropriate diluent. All similar samples were also run at this dilution.

Materials

96 well Maxisorp plate

200ml coating buffer containing 0.318g $Na_2CO_3$, 0.596g $NaHCO_3$ (pH 9.6)

Capture antibodies (Table 2-1, all purchased from Abcam unless otherwise stated)

Clingfilm

Plate washer

Wash buffer: 1x PBS containing 0.05% Tween 20

Blocking buffer: 5% protein in wash buffer (Table 2-1)

Standards (Table 2-1)

Detection antibodies (Table 2-1, all purchased from Abcam unless otherwise stated)

12ml OPD solution: 2 OPD tablets, 6µl hydrogen peroxide

Aluminium foil

1M sulphuric acid

Spectrophotometer at 492nm

Method

For each ELISA, the following steps were performed. Table 2-1 overleaf provides details for each specific protein ELISA.

The capture Ab was diluted in coating buffer and 100µl pipetted into each well of the 96-well plate, covered with clingfilm and incubated (see Table 2-1). Capture Ab was aspirated and plates washed 3 times with 200µl washing buffer using the plate washer. 100µl blocking buffer was added, plate covered with clingfilm and incubated (see Table 2-1). A standard curve for each protein was prepared using serial dilution (200-6.25ng/ml). Samples were diluted as necessary. 100µl standard and sample was added to the plate. The plate was covered with clingfilm and incubated for 90 minutes at 37°C. The plate was washed as described above and detection Ab diluted in blocking buffer and 100µl added to each well (see Table 2-1). The plate was covered in clingfilm and incubated for 1 hour at room temperature. Detection Ab was aspirated and plate washed 5 times as described above. 100µl OPD solution was added to each well at

timed intervals, plate covered in foil and incubated until orange colour developed. The reaction was stopped by addition of 50µl acid to each well at timed intervals. Absorbance at 492nm was quantified.

**Table 2-1. Hepato-specific protein ELISA methodology**

| | | Albumin | Alpha-1-fetoprotein | Alpha-1-antitrypsin | Alpha-1-acid glycoprotein | Fibrinogen |
|---|---|---|---|---|---|---|
| Capture Antibody | Cat. # | Dako A0001 | Ab10071 | Dako A0012 | Dako A0011 | ab6666 |
| | Dilution | 1/1000 | 1/1000 | 1/790 | 1/1000 | 1/1000 |
| | Incubation | 1h at RT | Overnight at 4°C | Overnight at 4°C | Overnight at 4°C | Overnight at 4°C |
| Blocking | Blocking buffer | 5% non-fat milk | 5% non-fat milk | 5% FCS | 5% non-fat milk | 5% non-fat milk (removed) |
| | Incubation | 1h at RT | 1h at RT | 2h at RT | 1h at RT | 1h at RT |
| Detection Antibody | Cat. # | ab24458-200 | Ab10072 | ab7635-s | Ab34720-10 | ab7539 |
| | Dilution | 1/4444 | 1/3500 | 1/2000 | 1/4000 | 1/1000 |
| | Incubation | 1h at RT | 1h at RT | 1h at RT | 1h at RT | 1h at RT |
| Standard | Antigen | Dako X0908 | Abcam ab38189 | Citrated plasma | Dako X0908 | Citrated plasma |

## 2.8. Quantification of broad-spectrum cytochrome P450 activity

7-Ethoxycoumarin (7-EC) was used as a substrate to determine broad spectrum cytochrome P450 activity as 7-EC-O-deethylation (ECOD) to form 7-hydroxycoumarin (7-HC) is catalysed by cytochrome P450 enzymes from CYP1, 2 and 3 families. Induction with indirubin was employed to increase cytochrome P450 function.

Materials

Indirubin (Biomol CC206-0005)

DMSO

HBSS

7-EC (Sigma E1379, 40mM in MeOH)

Complete culture medium

Serum-free culture medium

75% trichloroacetic acid (TCA)

7-HC (Sigma H2400) (160mM in 1M NaOH)

Chloroform

0.01M NaOH/1M NaCl solution

1.5ml microfuge tubes

Vortex

Clear bottomed plates

Cytoflour 4000 fluorescence platereader (PerSeptive Biosystems)

Method

**2.8.1. Induction using indirubin**

A 20mM stock (1000x) of indirubin in DMSO was prepared and stored at -20°C in single use aliquots until required. Cells were washed twice with HBSS and indirubin diluted to 20µM (1x) in complete culture medium and incubated for 24 or 48 hours as indicated in later chapters.

**2.8.2. ECOD assay**

7-EC was diluted 1/200 to 200µM in pre-warmed serum-free complete medium. Cells or beads were washed twice with serum-free culture medium and 7-EC added to cultures. Plates were incubated under normal culture conditions for 3 hours, unless otherwise indicated, before 300µl conditioned medium was removed and transferred into a microfuge tube containing 7.5µl 75% TCA. A standard curve was prepared from 7-HC (50-1.5625µM) in serum-free media containing 200µM 7-EC. 7-HC was extracted by addition of 900µl chloroform, thorough mixing and vortexing. Samples were centrifuged at 3000g for 1 minute. The product was back extracted by transferring 500µl of the lower organic phase to a new 1.5ml microfuge tube containing 500µl 0.01M NaOH/1M NaCl. This was mixed well, vortexed and centrifuged at 3000g for 1 minute. 200µl of the upper aqueous phase was transferred to a 96-well plate and fluorescence was quantified at an excitation wavelength of 370nm and an emission wavelength of 450nm.

## 2.9. Cryopreservation of alginate-encapsulated liver cell spheroids

### 2.9.1. Use of controlled rate freezers

Controlled rate freezers (CRFs) were used to allow greater control of freezing rate during cooling. 2 CRFs were used throughout this thesis and which was used for which experiment will be indicated in the following chapters. Both CRFs rely on a nitrogen vapour supply via a solenoid valve in a feedback loop for cooling. A third CRF based on a different physical principle of cooling was also used and will be described separately.

Materials

Liquid nitrogen supply (for storage in vapour phase)

CRF nitrogen Dewar

R204 cell freezer and canes (Planer)

Kryo10 and baskets (Planer)

Method

2.9.1.1. Filling the CRF nitrogen Dewar

Prior to a cooling run, the CRF nitrogen Dewar was filled with liquid nitrogen. The pressure release valve on the Dewar was opened to release pressurised gas before filling. The central column was removed and Dewar filled from the main liquid nitrogen storage tank. Once filled, the central column was replaced and pressure release valve closed.

2.9.1.2. Entering and running a programme

Programmes were entered as per the manufacturers' instructions for each CRF. The capabilities of each CRF are given in Table 2-2 below.

**Table 2-2. R204 and Kryo 10 CRF capabilities**

| CRF | Cooling rates (°C/min) | Warming rates (°C/min) | Working range (°C) | Capacity (1.8ml cryovials) |
|---|---|---|---|---|
| R204 cell freezer | -0.1 to -35 | - | -150 to 50 | 24 |
| Kryo 10 Freezer | -0.01 to -50 | 0.01 to 10 | -180 to 50 | 726 |

The decant valve on CRF Dewar was opened and pressure adjusted to between 0.3 and 0.5 bar using the pressure raising coil if required. Once the desired start temperature was reached, samples were loaded into the chamber of the CRF and cooling profile run. Throughout the run, chamber temperatures were observed using the chart writer and/or additional thermocouples to ensure that the CRF was able to apply the desired cooling profile.

### 2.9.2. Cryopreservation and Thawing of ELS

Cryopreservation protocols for ELS were trialled after ELS had been cultured for 8 days following either Inotech encapsulation and static culture or 11 days after JetCutter encapsulation and FBB culture. Inotech-encapsulated ELS contained approximately $8x10^6$ nuclei/ml alginate. Each alginate bead contained 20-25 spheroids which contained ~25 cells each.

JetCutter-encapsulated cells typically contained up to $50x10^6$ nuclei/ml alginate. Each alginate bead contained 20-25 spheroids which contained between 100 and 150 cells each. For the majority of this thesis, Inotech-encapsulated were used but JetCutter ELS were used on occasion and when they were, this is indicated in the Methods section of that Chapter.

#### 2.9.2.1. Addition of CPA

Materials

DMSO

UW solution

1.8ml cryovials

Ice

Method

A 15% v/v DMSO in UW solution (CPA mixture) was prepared on ice and kept on ice until required. ELS were resuspended 1:4 in CPA mixture (to give a final concentration of 12% DMSO) and mixed well on ice. This mixture was aliquotted into cryovials on ice in 1.25ml volumes (0.25ml ELS and 1ml CPA mix). ELS were exposed to CPA on ice for approximately 15 minutes before initiation of cooling.

2.9.2.2. Starting cryopreservation protocol

The starting cooling profile for this thesis is identical to a cooling profile that has previously been shown to be optimal for cryopreservation of single cell suspensions of human hepatocytes. This profile is designed to apply a slow cooling rate of approximately 2°C per minute. The authors recognise that supercooling is likely to occur and so included a shock-cooling step, designed to trigger nucleation without supercooling. Following this shock cooling step are 2 further ramps designed to counteract the release of latent heat of crystallisation and continue sample cooling at a slow controlled rate of 2°C/minute. This was determined by the authors empirically. The CRF chamber is cooled to 4°C before cooling began to ensure that samples were not exposed to CPA at warm temperatures where damage may occur. Ramp 2 is the shock-cooling step designed to trigger nucleation (Diener et al. 1993). The full cooling profile is given below:

Ramp 1: -2°C/min to -8°C

Ramp 2: -35°C/min to -28°C

Ramp 3: -2.5°C/min to -33°C

Ramp 4: +2.5°C/min to -28°C

Ramp 5: -2°C/min to -60°C

Ramp 6: -10°C/min to -100°C

Ramp 7: -20°C/min to -160°C

2.9.2.3. Storage of cryopreserved samples

Materials

Liquid nitrogen supply

Biostor 5 (Statebourne)

Method

Upon completion of the cooling profile, samples were rapidly transferred to nitrogen storage within a Biostor 5. Samples were normally stored in the vapour phase of liquid nitrogen. The Biostor 5 was topped up with liquid nitrogen every 2-3 days to ensure that cryogenic storage temperatures were maintained. Samples were normally stored for at least 1 week, and never less than 24 hours, before thawing.

2.9.2.4. Thawing of ELS following cryopreservation

Materials

37°C water bath

Ice

Iced complete medium

Warmed complete medium

50ml centrifuge tubes

Timer

Method

Cryovials were thawed using a 37°C water bath until the last ice crystal had melted before the vials were returned to ice. The contents of the cryovials were pooled on ice into a centrifuge tube, alginate beads were allowed to settle and CPA media was removed. Iced medium was added on ice slowly over 12 minutes, then removed and replaced with warmed culture medium and ELS were returned to culture.

For example, if 6 cryovials containing a total of 1.5ml ELS were thawed, 250µl iced medium was added each minute for the first 3 minutes, 500µl iced medium added each minute over the next 3 minutes, a total of 3ml over the next 3 minutes and a total of 6ml over the final 3 minutes. These steps were performed on ice to minimise injury due to DMSO cytotoxicity. The beads were then allowed to settle and iced medium removed. Warmed medium was then used to resuspend the beads in a ratio of 1:32 (beads:medium) before they were returned to culture. This protocol was designed to minimise osmotic excursions in ELS. When more or fewer vials were thawed, this protocol was scaled up or down as appropriate.

**2.9.3. Temperature measurements during cryopreservation**

During cryopreservation, it was possible to measure and log the temperature of both chamber and samples using thermocouples and a data logger to provide assurance that the CRFs were able to apply the cooling profile, observe nucleation events and evaluate heat transfer.

Materials

Type K thermocouple (Pico Technology)

TC-08 data logger (Pico Technology)

Laptop

PicoLog recorder software (Pico Technology)

Specially adapted cryovials

Method

Thermocouples were connected to the data logger which was connected via USB port to a laptop. Log settings were applied, typically 10 readings per second for the duration of the cooling profile. Thermocouples were placed either into the CRF chamber or into specially adapted cryovials (see Figure 2-5) for sample temperature measurement.

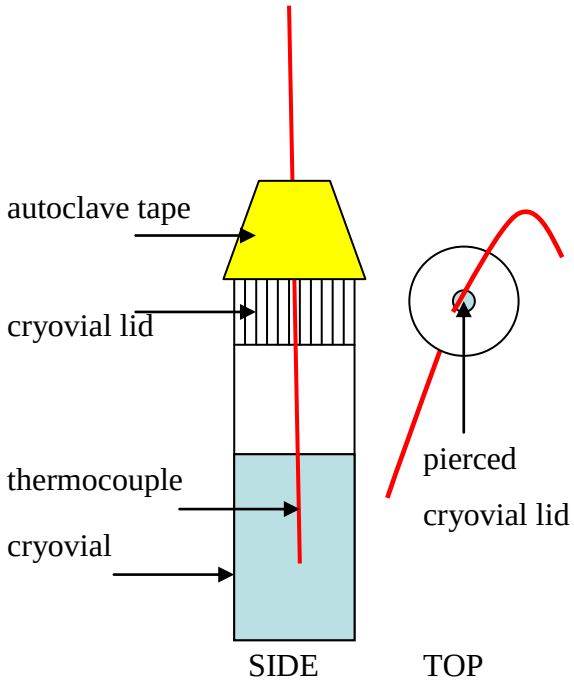


**Figure 2-5. Specially-adapted cryovials for sample temperature measurement.**

Data was logged using PicoLog software and exported into Excel for analysis.

## 2.10. Differential scanning calorimetry

Differential scanning analysis (DSC) is used to identify exo- or endothermic reactions, such as glass transitions, melting and nucleation events, allowing definition of biophysical characteristics of cryopreservation media used in the studies. In heat flux DSC, as here, 2 pans are subjected to a cooling and heating cycle over the temperature range of interest. One pan contains the sample of interest, of known mass. The other pan, the reference pan, is empty. The temperature of both pans is monitored using thermocouples and kept equal by addition of heat to either pan as required. This data is then represented on a DSC trace which plots heat flow (Watts) against temperature (°C),

allowing thermal properties to be observed. Some typical thermal events and how they appear on a DSC trace are shown in Figure 2-6.

The heating rate applied during DSC is important. Generally, slow warming rates allow better separation of thermal events occurring at similar temperatures. However, faster warming rates can amplify transitions. Modulated DSC (mDSC) is a technique in which a sine wave modulation is introduced on top of a linear heating rate. By applying this, small thermal events can be identified even at relatively rapid heating rates. mDSC was used here.

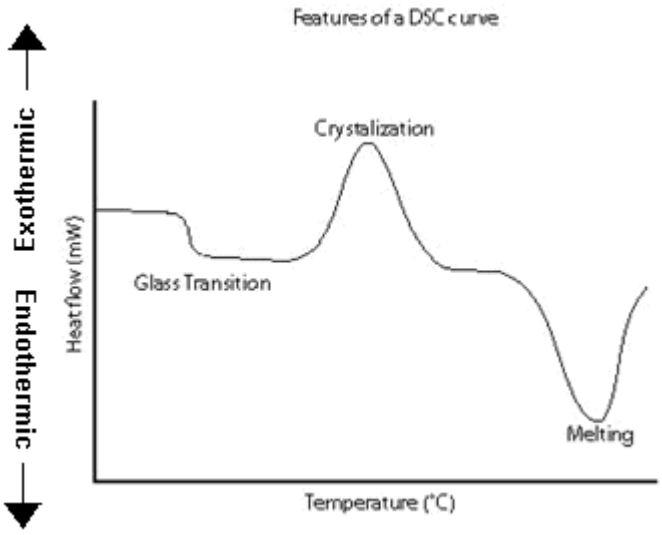


**Figure 2-6. Schematic of a DSC trace showing common thermal events.**

Modified from "Features of a DSC curve". Materials

Q2000 Calorimeter (TA Instruments)

High volume stainless steel pans

Universal Analysis software (TA Instruments)

Balance

Crimper

Method

Empty pans for the samples were weighed before 80µl sample was loaded into each. These pans were then crimped, reweighed and sample mass calculated. The reference pan was loaded onto the calorimeter in auto-sampler position 1. Samples were loaded sequentially in the following positions. The mass of each sample was entered into the software. Analysis was at 5°C/min with modulation of 1°C/min. Data was analysed using Universal Analysis software.

## 2.11. Evaluation of oxidative stress

### 2.11.1. Reactive oxygen species assay

Reactive oxygen species (ROS) can be quantified using dichlorohydrofluorescein diacetate (DCF). DCF is cell-permeable but once located intracellularly, is cleaved to an ionic moiety. In the presence of hydrogen peroxide, it is oxidised to form a fluorescent product, see Figure 2-7.

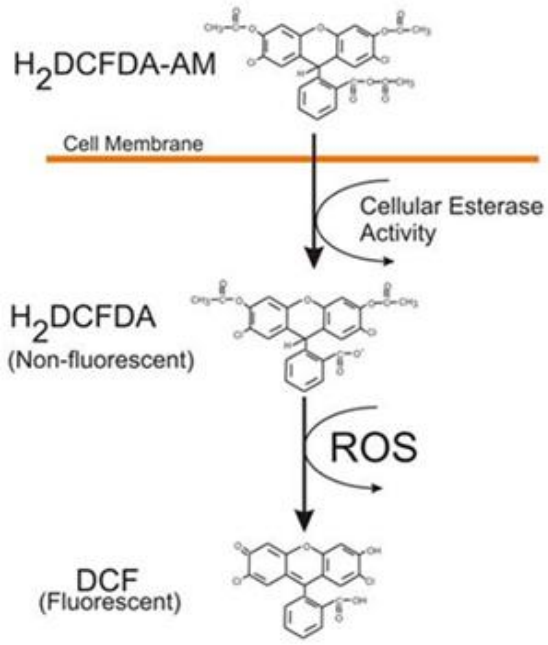


**Figure 2-7. Formation of fluorescent product, DCF, by ROS.**

**Taken from "Formation of fluorescent product DCF by ROS".**

Materials

DCF (Sigma 35848) (50mM in ethanol)

Hydrogen peroxide

Serum- and phenol-red free medium

Microfuge tubes

96-well clear-bottomed plates

Cytoflour 4000 fluorescence platereader (PerSeptive Biosystems)

Method

2.11.1.1.Method for 3D cultures

A 50mM DCF/ethanol stock was prepared and stored at -20°C until required. A standard curve was prepared using hydrogen peroxide by serial dilution in medium (50-

1.6mM) and 50µl pipetted into a 96-well plate in duplicate. A medium/DCF/empty bead blank was also included. Beads containing ~$1x10^5$ cells were washed twice with medium pipetted into the wells in 50µl volumes. DCF was diluted 1 in 1000 in medium to a final concentration of 50µM and 50µl of this added to the cells. Fluorescence was measured at an excitation wavelength of 485nm and emission wavelength of 530nm. ROS concentration was determined from the standard curve.

2.11.1.2.Method for monolayer cultures

A 50mM DCF/ethanol stock was prepared and stored at -20°C until required. A standard curve was prepared using hydrogen peroxide by serial dilution in medium (50-1.6mM) and 50µl pipetted into a 96-well plate in duplicate. A medium/DCF blank was also included. Cells were washed twice with medium and 50µl fresh medium added. $1x10^5$ cells per well were used. DCF was diluted 1 in 1000 in medium to a final concentration of 50µM and 50µl of this added to the cells. Fluorescence was measured at an excitation wavelength of 485nm and emission wavelength of 530nm. ROS concentration was determined from the standard curve.

### 2.11.2. Oxidative damage assay

Malondialdehyde (MDA) is a lipid peroxidation product which may be measured using thiobarbituric acid reactive substance (TBARS) assay. MDA reacts with TBA to form a fluorescent product.

Materials

Serum- and phenol red-free medium

Malondialdehyde

TBA reaction mix: 0.375% TBA, 0.25M hydrochloric acid, 0.01% butylated hydroxyl toluene

N-butanol

Microfuge tubes

Vortex

Heating block

21G needle

96-well clear-bottomed plates

Cytoflour 4000 fluorescence platereader (PerSeptive Biosystems)

Method

A standard curve was prepared from MDA by serial dilution in medium (50-0.8µM). A medium blank was also included. Conditioned medium was collected from samples, normally after 4 hours unless otherwise stated. 250µl samples and standards were transferred into new microfuge tubes. 250µl TBA reaction mix was added to each tube, before vortexing to mix thoroughly. The lid of each tube was pierced using a needle and tubes heated to 95°C for 45 minutes. Samples were left to cool before 500µl butanol was added and vortexed again. Samples were centrifuged at 1250g for 10 minutes before 100µl of the upper organic layer was transferred to a 96-well plate in triplicate. Fluorescence was measured at an excitation wavelength of 530nm and emission wavelength of 580nm. MDA concentration was determined from the standard curve.

### 2.12. Statistical analysis

Statistical analysis was performed using Microsoft Excel package from MS Office (2007). Comparisons between two values were made using Student's t test (two-tailed). One-way ANOVA was chosen when comparing more than two values. Unless stated otherwise, values in text, tables and figures were expressed as the mean ± standard deviation (SD) and *n* numbers are given in the accompanying text. Generally, $p<0.05$ was considered to be significant. In Tables and Figures, p values are expressed as * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.005$).

# CHAPTER 3

# Cooling rates, Supercooling and Nucleation

## 3.1. Introduction

The aim of this thesis was to develop and optimise a cryopreservation protocol for encapsulated liver cell spheroids (ELS) that would ultimately comprise the cellular component of a bioartificial liver (BAL) for treatment of acute liver failure (ALF). Cryopreservation will be required to treat ALF due to the rapid and unpredictable onset coupled with the fact that ELS require 8-9 days of culture to reach performance-competency (i.e. in sufficient number with sufficient function to treat ALF).

### 3.1.1. Time over which ELS should be characterised in post-thaw cultures

As ELS are needed to treat ALF, characterisation of the recovery of, and the time over which, this occurs for ELS post-cryopreservation is necessary. To effectively manage ALF, treatment should begin soon after diagnosis (although not necessarily immediately allowing time to ensure that diagnosis is correct and allow a baseline for that patient to be established and be sure that treatment with a BAL is absolutely necessary). Therefore, it is estimated that ELS should be optimally functional beyond 24 hours post-cryopreservation and certainly out to 48 hours, although if function were maintained beyond this time to 72 hours, this would be useful and, for the purposes of this thesis, could confirm recovery is sustained.

### 3.1.2. Cooling rates, supercooling, and warming rates

As described in Chapter 1, avoiding the damaging and potentially lethal effects of intracellular ice formation (IIF) and solute toxicity, that arise from application of incorrect cooling rates during cryopreservation, is vital to conserve ELS functionality. Further to this, is the risk of supercooling. Supercooling can result in a higher effective cooling rate than intended which will often result in IIF and poor recovery of ELS in post-thaw cultures.

The optimal warming should also be determined. Recrystallisation of small ice crystals to larger, more thermodynamically stable crystals, should be avoided. Recrystallisation is likely to occur if samples are warmed slowly. Recrystallisation during rapid warming is less likely as ice melts rapidly to water, before recrystallisation can occur.

## 3.2. Aims

The first aim of this Chapter is to characterise ELS following cryopreservation out to 72 hours using a standard hepatocyte cryopreservation protocol that has previously been demonstrated to be optimal. This will establish a baseline recovery upon which improvements would be made.

Methods of improving recovery will then be investigated. This will be achieved by determining if and how much supercooling is occurring and how damaging this is to ELS. If supercooling is occurring, then methods to reduce supercooling will be investigated and effects of limiting supercooling on ELS will be shown. In addition, the optimal warming rate will be determined.

## 3.3. Methods

### 3.3.1. Determination of optimal cooling rate for ELS

For all ELS, the starting profile began at 4°C followed by cooling at 2°C per minute to -8°C (chosen as a low non-freezing temperature). Below -8°C, samples were cooled at a range of rates, as described in sections 3.3.1.1 and 3.3.1.2 to -60°C. Below -60°C, samples were cooled at 10°C/min to -100°C, then -20°C/min to -160°C before storage in the vapour phase of liquid nitrogen. These profiles were based upon that described in section 2.9.2 of the previous chapter (Diener et al. 1993). The rate of cooling between -8°C and -60°C is most relevant to ELS recovery as this is the temperature range between which extracellular ice will form and ELS will or will not suffer the effects of either solute concentration or intracellular ice.

3.3.1.1. Preliminary study

Beyond -8°C, ELS were cooled at either -2, -10, -20 or -30°C/min using the R204 cell freezer. Finally, a cohort were snap frozen in liquid nitrogen achieving a cooling rate of approximately 500°C/min between 4°C and –170°C.

3.3.1.2. Subsequent studies

Beyond -8°C, ELS were cooled at either -1, -2 or -3°C/min using the R204 cell freezer.

### 3.3.2. Determination of supercooling

This is an appropriate but quick and easy way to assess supercooling as long as other variables (sample size, container, cooling rate) are standardised. True measurements of the equilibrium melting point require the use of a differential scanning calorimeter (see section 2.10). Supercooling was normally calculated as the difference between the minimum temperature reached prior to nucleation (i.e. the temperature at point 3 in Figure 3-1) and the warmest temperature achieved by the sample after this time. Supercooling was expressed in °C. This method has previously been described (Salin et al. 1998) and provides a simple way to determine supercooling.

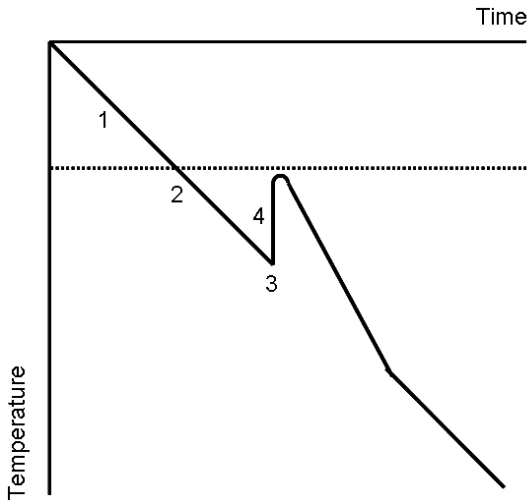


**Figure 3-1. Typical time-temperature sample cooling profile.**

**This schematic shows how sample supercooling can be observed on a time temperature profile (solid line). The equilibrium melting point of the sample is also shown (dashed line). 1 shows sample cooling above equilibrium melting point. At 2, sample is cooled below the equilibrium melting point (i.e. is supercooling). 3 is when nucleation/ice formation occurs. 4 shows the release of latent heat of crystallisation resulting in an increase is sample temperature towards the equilibrium melting point.**

#### 3.3.2.1. Cooling profiles

For all supercooling studies, the Diener cooling profile (Diener et al. 1993) was used as this has been previously demonstrated to be optimal for suspensions of human hepatocytes and was the "starting" protocol for this thesis.

#### 3.3.2.2. CPA media

For all supercooling studies, Celsior solution (Sangstat, Lyon, France) was used as a vehicle solution for DMSO. The final DMSO concentration used was always 12%. Although UW solution is considered to be the gold standard for hepatocyte cryopreservation (Feng et al. 2007), I found that nucleation temperatures were extremely variable when UW was used. This is in agreement with others where nucleation temperatures using a slow cooling profile ranged from -8°C to -24°C when UW was used (Diener et al. 1993). However, Celsior solution resulted in less variable nucleation temperatures, producing a more reliable data set and this is the reason for its use in the nucleation studies described in this Chapter. Reasons for this improvement over UW solution with respect to variability are likely due to the chemical constituent

differences, for example, UW solution contains considerably more starch than Celsior solution.

### 3.3.3. Determination of equilibrium melting point by mDSC

An alternative (and more accurate) method for determining the equilibrium melting point using DSC was also used on occasion, when greater accuracy was required. This method could not be used at all times as the equipment required was not available in-house.

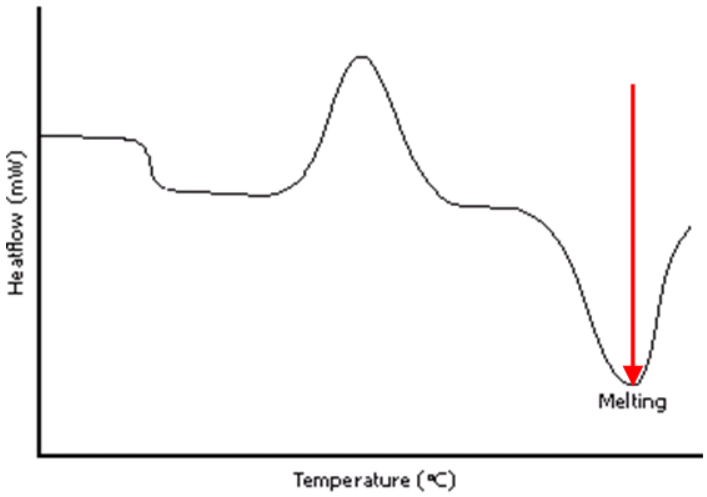


**Figure 3-2. Determination of equilibrium melting point using differential scanning calorimetry.**

**The equilibrium melting point was determined from the melt curve and was defined as the temperature at which the trough was greatest (indicated by the red arrow).**

Modulated differential scanning calorimetry (mDSC) was used to find the equilibrium melting point using the methods described in Chapter 2, section 10. The equilibrium melting point was determined from the melt curve. The temperature at the trough of the melt curve is the equilibrium melting point (indicated with a red arrow in Figure 3-2 above. In the method described in section 3.3.2 above, the sample temperature is unlikely to return the true equilibrium melting point (even though it theoretically should do so) as the chamber temperature, which will be lower than the sample temperature, will cool the sample temperature below the equilibrium melting temperature. By using mDSC and looking at the melting point (which is identical to the equilibrium melting point) this phenomenon is avoided and the true equilibrium melting point can be accurately determined.

Supercooling was then calculated as the difference between the equilibrium melting point (determined using mDSC) and the temperature of nucleation (determined using thermocouples).

### 3.3.4. Silver iodide alginate bead formation

Silver iodide is a known nucleating material (Finnegan 1998) and has been successfully used to reduce supercooling during cryopreservation of sperm and embryos (Chen et al. 1993;Kojima et al. 1988). Silver iodide alginate beads were formed using a method adapted from Horak et al (Horak et al. 1998).

Materials

Alginate beads without cells (produced as described in Chapter 2, section 2.2.1)
Potassium iodide (0.07g/ml)
Iodine (0.045g/ml)
Silver nitrate (30% w/v)
Ethanol
Petri-dish
Aluminium foil

Method

Alginate beads were immersed in a 1:1 aqueous-ethanolic solution of potassium iodide and iodine for 48 hours in a petri-dish. Beads were washed twice and immersed into silver nitrate solution for 24 hours in the dark.

### 3.3.5. Cryomicroscopy

Cryomicroscopy can be used to observe samples at low temperatures. Formation of ice following nucleation can be seen both intra- and extracellularly. The presence of ice is typically characterised by blackening, or "flashing", as the ice crystals scatter incident light although when very rapid or very slow cooling rates are applied this is not always true. Cryomicroscopy can also show the location of ice within or around samples and growth of ice crystals can be observed. Cryomicroscopy can also show cellular cryo-dehydration as cells dehydrate by exosmosis in response to increasing concentrations of extracellular solutes (Grout & Morris 1987).

Cryomicroscopy was performed within the Bio-Imaging and Assay Development Group at the National Institute for Biological Standards and Control (NIBSC) under the supervision of Dr. Roland Fleck.

Materials

Alginate beads containing ELS

University of Wisconsin solution (UW)

Cryopreservation (CPA) media (15% DMSO in UW (v/v))

Cholesterol

Propidium iodide (PI)

Linkam FDCS196 cryostage

Nitrogen mini-Dewar

Circular glass slide

Cover slip

Metal shim

JVC KY-F55BE camera

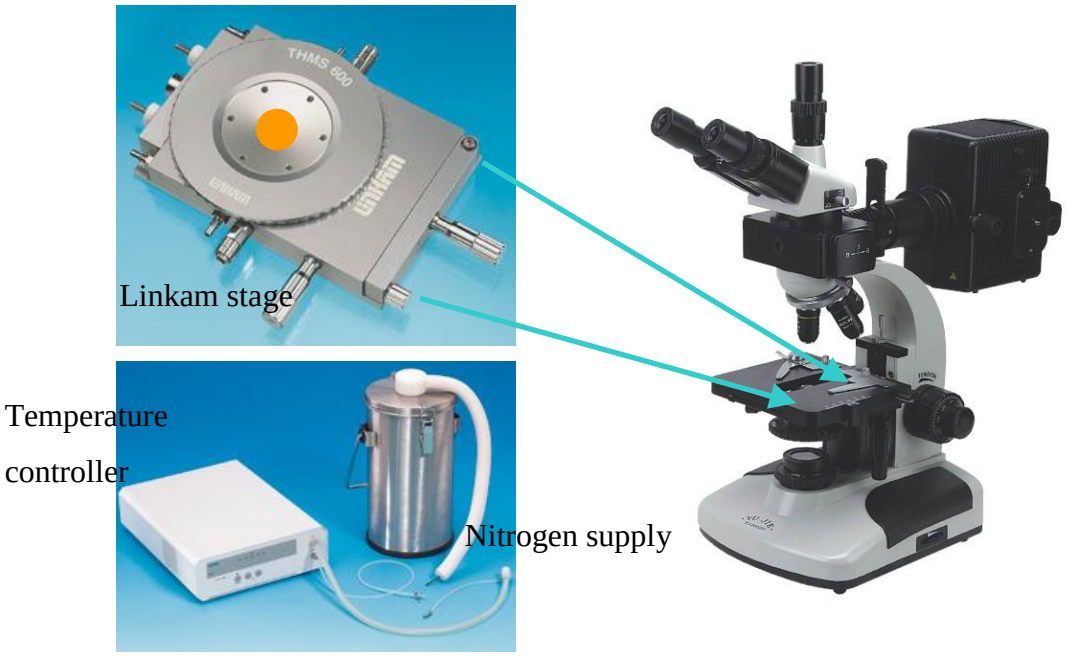


**Figure 3-3. Cryomicroscope setup.**

**Samples were loaded onto the Linkam stage in the position indicated by the orange circle. The Linkam stage was loaded onto the microscope stage. The Linkam stage was cooled using a nitrogen supply and temperature controlled during cooling.**

Method

ELS were prepared for cryomicroscopy by mixing with either UW solution or with CPA media in a ratio of 1 to 4 as described Chapter 2, section 2.9.2 to achieve a final DMSO concentration of 12%. Cholesterol was added to some samples at a concentration of 1.1mg/ml. 20µl PI was added to capture fluorescent images. Which treatment ELS

received will be indicated in the results section of this Chapter. 100µl samples were loaded onto the slide and a single shim placed around the sample. A cover slip was added and slide loaded onto the cryostage. Images and videos were captured using a JVC camera using Linksys32 software. Microscope setup is shown in Figure 3-3. Linksys32 software was used to cool samples using a standard profile as below, always beginning at ambient temperature:

Ramp1: 1°C/min to 20°C

Ramp 2: 2°C/min to -40°C

Ramp 3: Hold for 5 minutes

Ramp 4: 5°C/min to 20°C

### 3.3.6. Thawing protocols

To achieve different warming rates, following cryopreservation, different thawing protocols were used.

Materials

37°C water bath

20°C water bath

4°C fridge

Method

The standard thawing protocol is described in Chapter 2, section 2.9.2.4 using a 37°C water bath and aimed to achieve rapid warming rates. However, slower warming rates were achieved by placing samples in either a 20°C water bath, 20°C in air or 4°C in air (using a 4°C fridge).

### 3.3.7. Assessing recovery in post-thaw cultures

The methods used to assess recovery in post-thaw cultures used were viability, cell number and function as described in Chapter 2. Cryopreserved ELS were compared to unfrozen ELS at the equivalent time point. For example, cryopreserved ELS that were thawed and returned to culture for 24 hours would be compared with unfrozen ELS that had been cultured for 24 hours, after the cryopreserved ELS were frozen.

## 3.4. Results

### 3.4.1. Cooling rate

3.4.1.1. Preliminary study

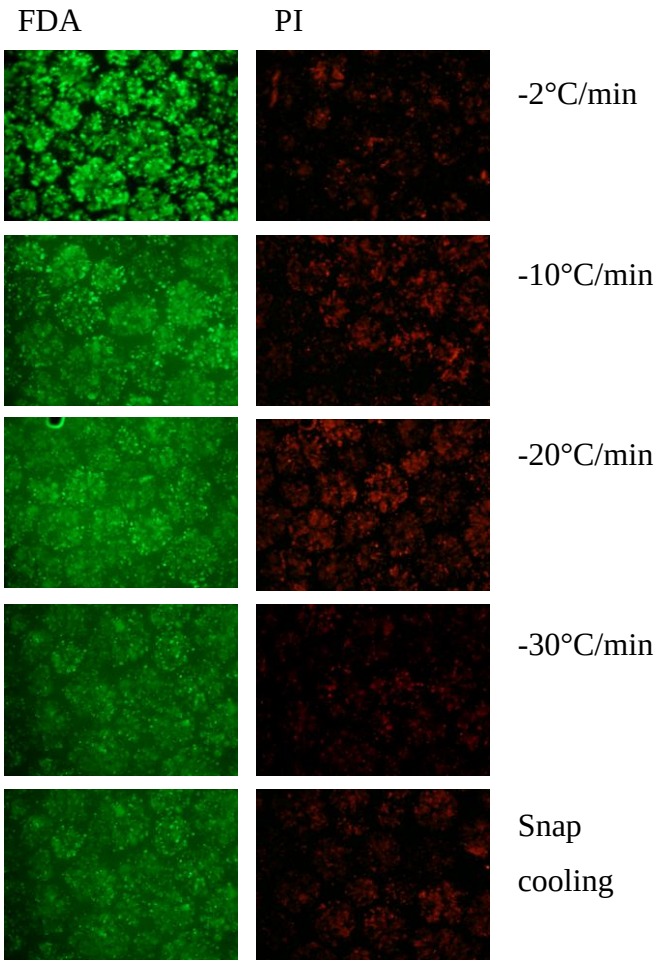


**Figure 3-4. Viability of ELS cooled at different cooling rates.**

**ELS were cooled at either 2, 10, 20 or 30°C/min between -8°C and -60°C (before more rapid cooling to -160°C) or snap cooled by immersion in liquid nitrogen (achieving an approximate cooling rate of -500°C/min between 4°C and -170°C). ELS were warmed rapidly using a 37°C waterbath. Micrographs show ELS stained with either FDA (live) or PI (dead). Exposure times are 64 and 250ms for FDA and PI respectively. Original magnification x4.**

When ELS were cooled at 2°C/min, FDA staining remained specific to ELS cytoplasm although PI was staining was also visible in a small proportion of the cells. At all higher cooling rates, FDA staining was not specific to ELS cytoplasm and appeared to "leak" out of the cells to give an abnormally high background fluorescence. PI staining

was also increased at all rates above 2°C/min. These results are shown in Figure 3-4 above.

3.4.1.2. Subsequent study

Having established that approximately 2°C/min was optimal for ELS, this was optimised using a narrower range of cooling rates (between 1 and 3°C/min) and recovery of ELS assessed. Viable cell numbers were comparable at all 3 rates at 24 and 48 hours post-warming but by 72 hours a statistically significant improvement was apparent when ELS were cooled at 2°C/min.

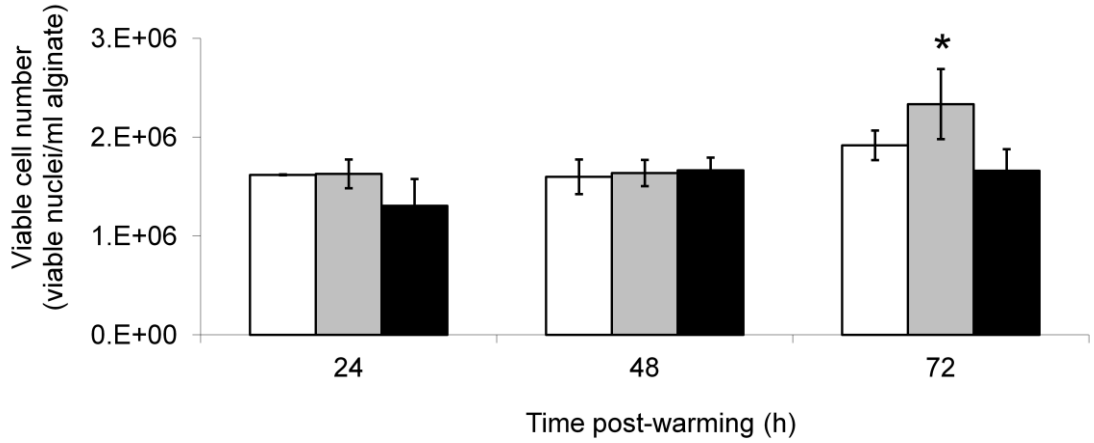


**Figure 3-5. Determination of optimal cooling rate for ELS.**

**ELS were cooled at either 1 (white bars), 2 (grey bars) or 3 (black bars) °C/min between -8°C and -60°C before more rapid cooling to -160°C. Vials were warmed rapidly using a 37°C waterbath. Viable cell numbers assessed at 24, 48 and 72 hours as indicated on x axis. n=5 wells +/- SD from a single experiment. *p<0.05 cf. ELS cooled at 3°C/min.**

### 3.4.2. Recovery of ELS in the first 6 hours following cryopreservation

3.4.2.1. Qualitative assessment of viability

To characterise ELS recovery after cryopreservation, viability of ELS was assessed in the first 6 hours following cryopreservation (Figure 3-6). FDA staining remained comparable between 0 and 6 hours indicating that ELS remained metabolically active. However, PI staining gradually increased between 0 and 6 hours indicating membrane permeability.

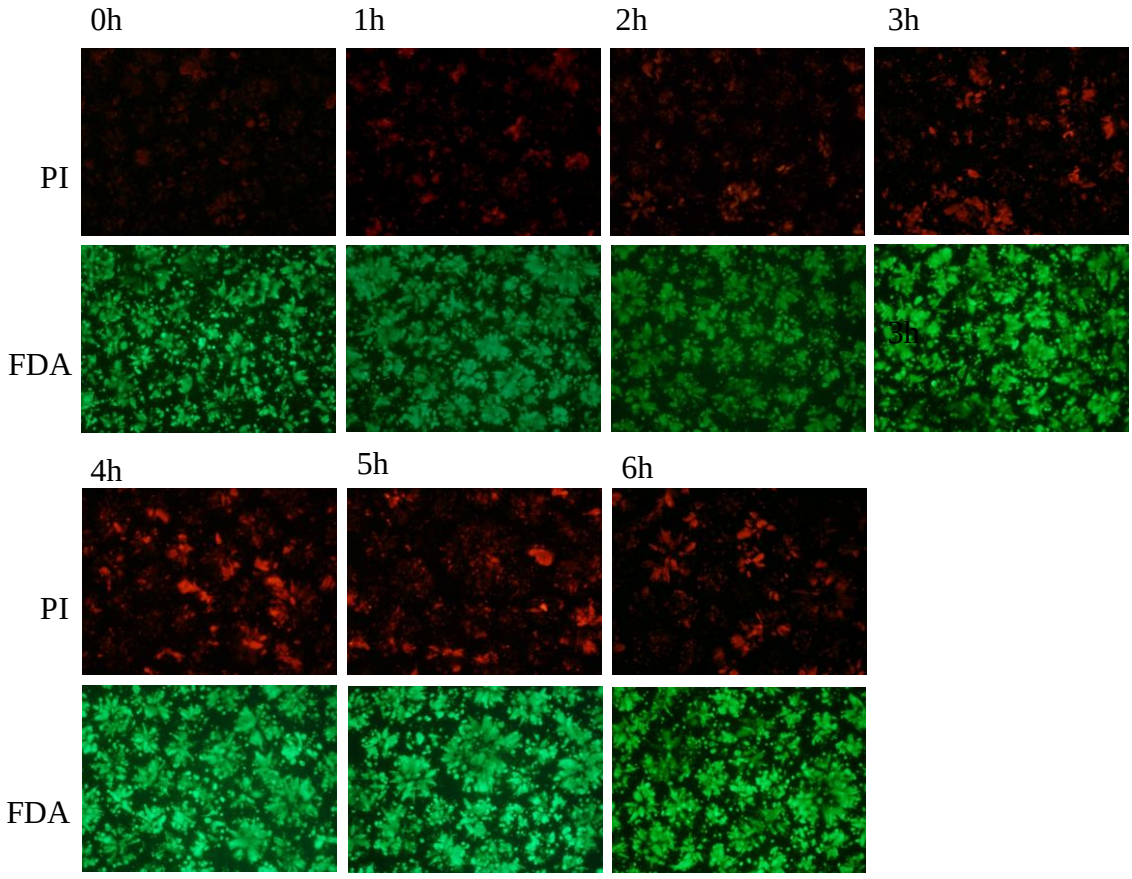


**Figure 3-6. Qualitative assessment of viability in the first 6 hours post-warming.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW. ELS were warmed rapidly using a 37°C waterbath and stained with FDA (live) and PI (dead cells) hourly from immediately after thawing out to 6 hours. The micrographs shown are typical of images from 4 separate experiments. Original magnification x4.**

### 3.4.2.2. Quantitative assessment of viability

The images captured were analysed using image analysis to quantify viability in the first 6 hours following cryopreservation. Immediately after thawing, viability was 80 +/- 16% which was not statistically significantly different to the pre-freeze control. However, quantified viability fell progressively over time and by 6 hours post-warming, viability was reduced to 45 +/- 18%, significantly lower than immediately post-warming, see Figure 3-7.

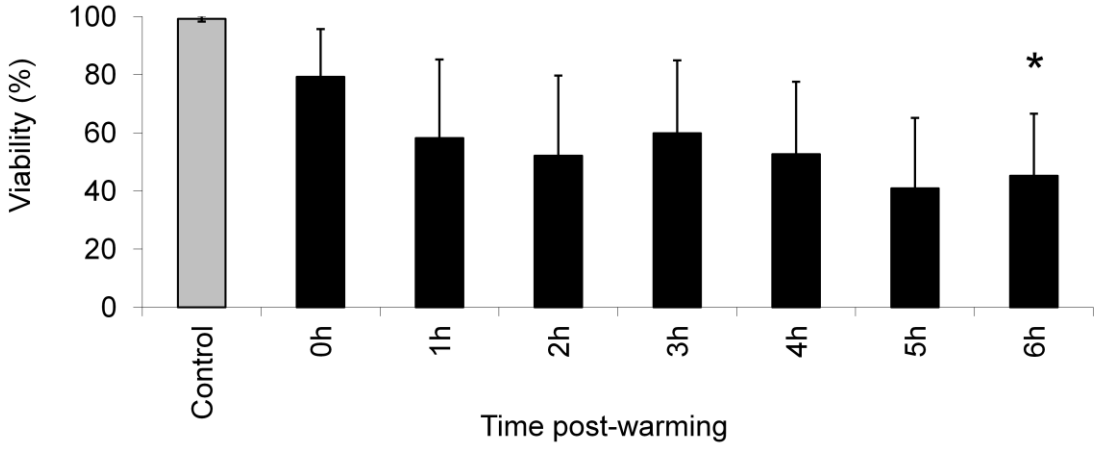


**Figure 3-7. Quantitative assessment of viability in the first 6 hours post-warming.**

**Data are n=4 +/- SD (from 4 separate experiments). Within each experiment, 5 fields were captured at each time point to obtain an average viability. Control indicates viability of a pre-freeze control. *p<0.05 compared to 0h post-warming.**

### 3.4.3. Recovery between 24 and 72 hours following cryopreservation

3.4.3.1. Qualitative assessment of viability

Viability was assessed at 24, 48 and 72 hours post-warming. PI staining was highest at 24 hours post-warming indicating membrane damage. However, beyond this time PI staining declined progressively to 72 hours. By 72 hours post-warming, only a few cells stained positive for PI indicating that most cells were membrane-intact, see Figure 3-8.

3.4.3.2. Quantitative assessment of viability

Viability was quantified from these images. On average, viability was 39 +/- 8%, 65 +/- 13% and 91 +/-1% at 24, 48 and 72 hours following cryopreservation respectively. Between 24 and 72 hours, average viability increased by 52%, see Figure 3-9.

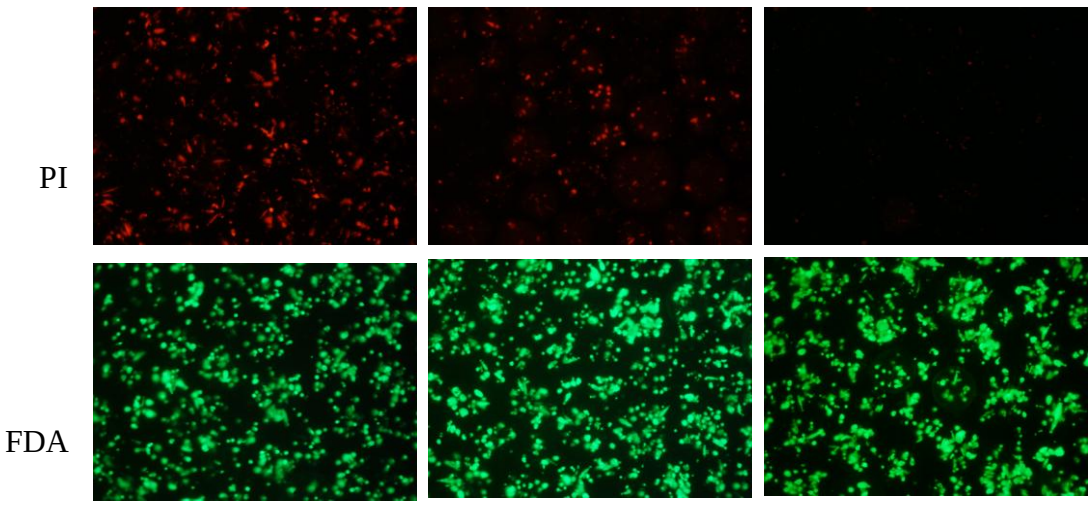


**Figure 3-8. Qualitative assessment of ELS viability between 24 and 72 hours post-warming.**

**ELS were cooled using a multistep cooling profile (Diener et al. 1993) in 12% DMSO/UW. ELS were warmed rapidly using a 37°C waterbath and stained with FDA (live) and PI (dead cells) at 24 (left), 48 (middle) and 72 (right) hours after thawing. The micrographs shown are typical of images from 5 separate experiments. Original magnification x4.**

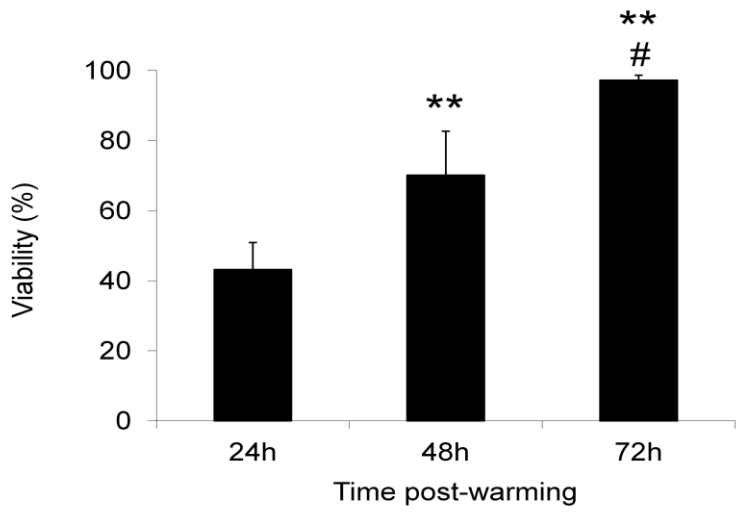


**Figure 3-9. Quantitative assessment of ELS viability between 24 and 72 hours post-warming.**

**Data are n=5 +/- SD (from 5 separate experiments). Within each experiment, 5 fields were captured at each time point to obtain an average viability. **p<0.01 cf. 24h, #p<005 cf. 48h.**

This recovery was considered to be insufficient for ELS to be used within a BAL. Supercooling was implicated as a possible cause of this low recovery following cryopreservation and was investigated next.

### 3.4.4. Supercooling measurements

#### 3.4.4.1. Supercooling without nucleators

In "dummy" vials containing 1.25ml cryopreservation mixtures, nucleation temperatures were -16°C +/- 9°C for 12% DMSO in UW solution and -22°C +/- 2°C for DMSO in Celsior solution. Although supercooling was greater in Celsior solution, nucleation temperatures were far more reproducible and reliable. These results are shown in Figure 3-10 below.

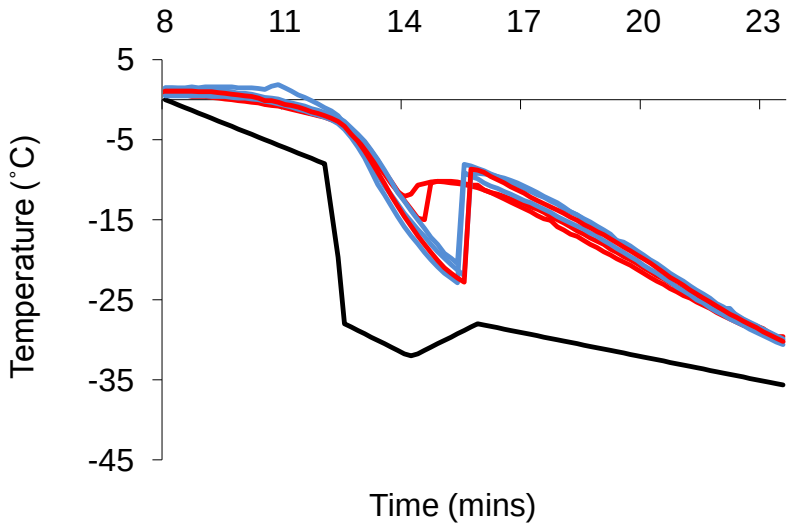


**Figure 3-10. Supercooling of common CPA media.**

**Nucleation is indicated by a spike towards the equilibrium melting point. The programmed cooling profile (Diener et al. 1993) is indicated by the black line. Temperature profiles of 12% DMSO in UW (red line) and 12% DMSO in Celsior (blue line) are also shown. Data from 3 separate runs for each CPA media are shown.**

#### 3.4.4.2. Effect of nucleators

##### *3.4.4.2.1. Silver iodide bead formation and effect on supercooling*

The process of silver iodide coating altered the appearance of alginate beads (Figure 3-11). Beads appeared less spherical and more wrinkled. Nucleation point using silver iodide coated beads in 12% DMSO in Celsior was -16 +/- 2°C (increased from -22 +/- 2°C in Celsior alone) (Figure 3-12).

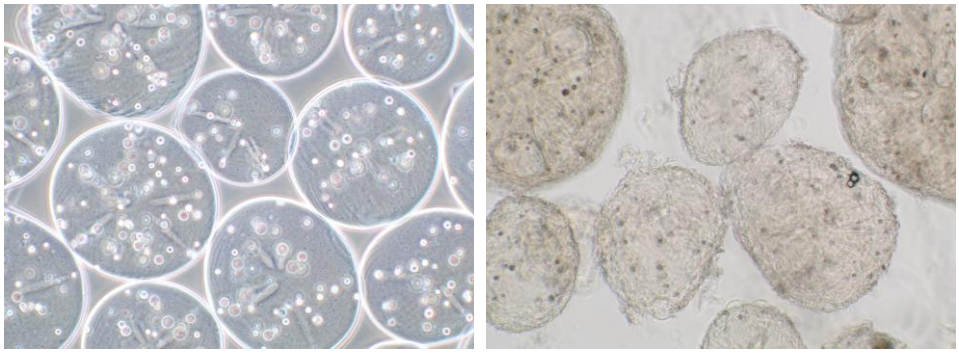

**Figure 3-11. Silver iodide bead formation.**

**Beads before silver iodide coating (left) and beads with silver iodide coating (right). Phase images. Original magnification x10.**

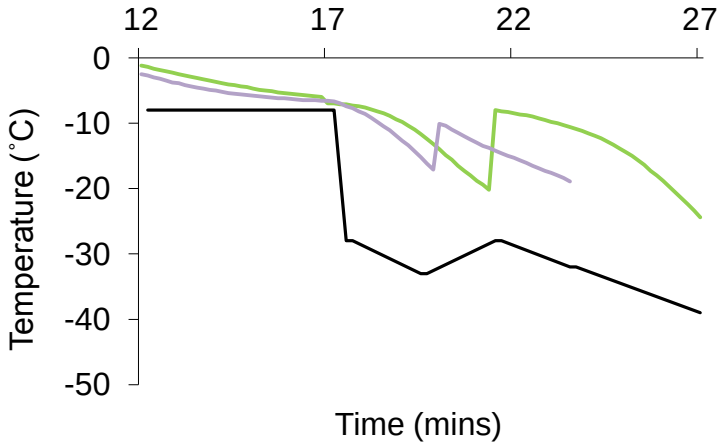


**Figure 3-12. Reduction in supercooling using silver iodide coated beads.**

**The programmed cooling profile (modified from Diener et al. 1993 to include a 5 minute hold at -8°C to ensure that the sample temperatures had stabilised) is indicated by the solid black line. Temperature profiles of samples of 12% DMSO in Celsior (green line) and 12% DMSO in Celsior with silver iodide coated beads (purple line) are also shown. Data from 1 run for each condition are shown (for ease of interpretation) but are representative of 2 further repeat experiments.**

Although silver iodide coated beads did increase nucleation temperature closer to the equilibrium melting point, since it was also found to have a detrimental effect on bead morphology, cholesterol was trialled to see if further improvements could be made.

*3.4.4.2.2. Effect of cholesterol on supercooling*

When cholesterol was included at 1.1mg/ml, nucleation occurred at -9.4°C +/- 0.7°C in 12% DMSO in Celsior (Figure 3-13). The equilibrium melting point was determined using mDSC and was -4.4˚C. The mDSC trace is shown in Figure 3-14.

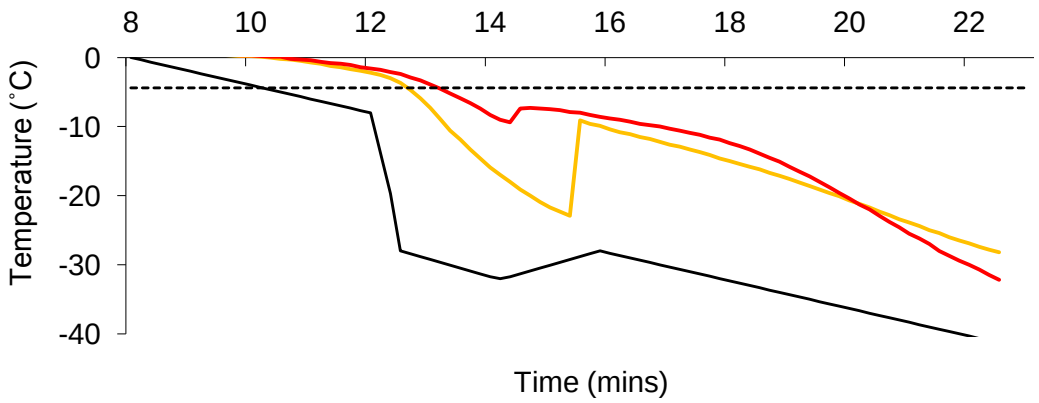


**Figure 3-13. Reduction in supercooling using cholesterol powder.**

**The programmed cooling profile (Diener et al. 1993) is indicated by the solid black line. Temperature profiles of samples of 12% DMSO in Celsior (orange line) and 12% DMSO in Celsior with cholesterol powder (red line) are also shown. Data from 1 run for each condition are shown (for ease of interpretation) but are representative of 2 further repeat experiments. The dashed line shows the equilibrium melting point which was determined using mDSC.**

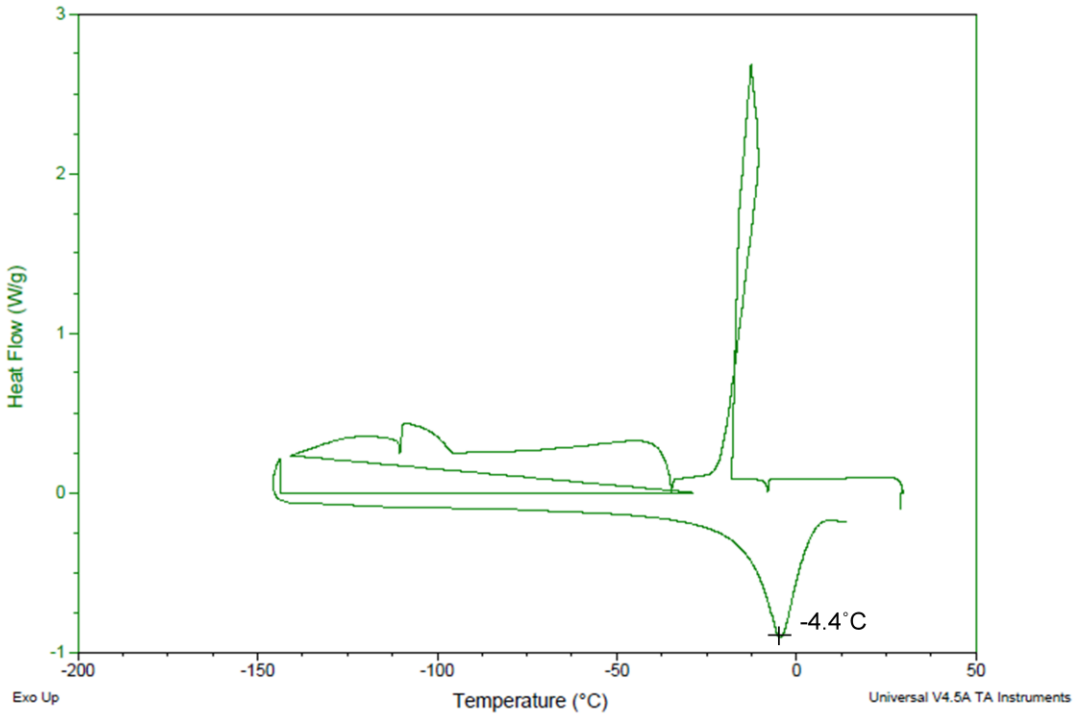


**Figure 3-14. Determination of equilibrium melting point using mDSC.**

**The equilibrium melting point was measured as -4.4˚C using mDSC.**

### 3.4.5. Effect of minimising supercooling using cholesterol on ice formation intra- and extracellularly

Cryomicroscopy was used to observe nucleation events and subsequent ice formation both inside and outside ELS. The cryomicroscope was unable to perform the shock-cooling step in the Diener cooling profile (Diener et al. 1993) and so a linear -2°C/min cool to -40°C was applied instead.

#### 3.4.5.1. Ice formation in the absence of DMSO

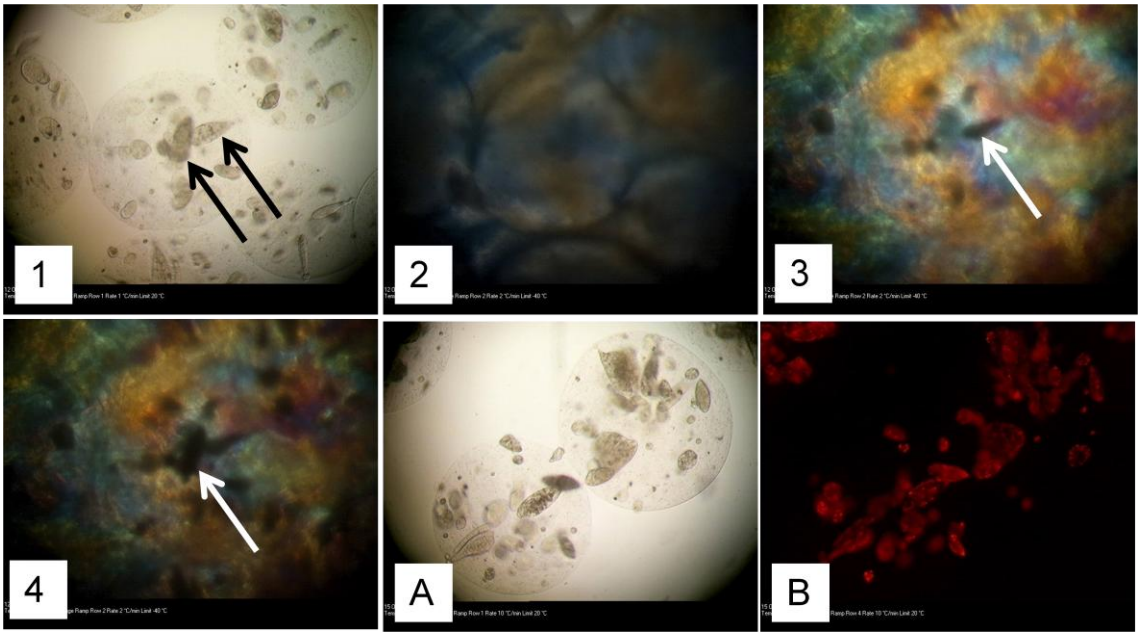


**Figure 3-15. Cryomicroscopy micrographs of ELS cooled without cryoprotectant.**

**Images 1-4 were captured during cooling. Temperatures when images were captured were room temperature, -16.4°C, -17.7°C and -20°C for images 1, 2, 3 and 4 respectively. White arrows show examples of spheroids that have formed intracellular ice (flashed) during cooling following extracellular ice formation. Image 1 shows the same spheroids before cooling. Image A was captured before cooling and image B shows the same ELS stained with PI and was captured at 20°C post-warming. Original magnification x4.**

When ELS were cooled without DMSO as a cryoprotectant, nucleation occurred at -16.4°C and was sudden, appearing as a black flash over the entire field (image 2 in Figure 3-15). By -17.7°C (image 3 in Figure 3-15) intracellular ice formation was apparent, characterised by darkening of cell spheroids within alginate beads. By -20°C (image 4 in Figure 3-15), all spheroids had darkened, indicating that ice was present intracellularly across the entire sample.

On a separate cooling run, under the same conditions (i.e. without cryoprotectant), ELS were also stained with PI. No PI staining was evident prior to cooling, indicating the membrane integrity was retained. However, after cooling (image B in Figure 3-15), all spheroids stained positive for PI establishing that membrane integrity had been compromised.

3.4.5.2. Ice formation in presence of DMSO

When ELS were cooled in 12% DMSO, ice nucleation occurred at a lower temperature of -20°C but again was sudden, appearing as a black flash across the entire field (image 2 in Figure 3-16). Immediately post-nucleation, there was no evidence of intracellular ice formation (image 3 in Figure 3-16) but when the sample was cooled to -22°C, some spheroids darkened indicating formation of intracellular ice (image 4 in Figure 3-16). When ELS were cooled to -40°C, there was no indication of any further intracellular ice formation (image 5 in Figure 3-16).

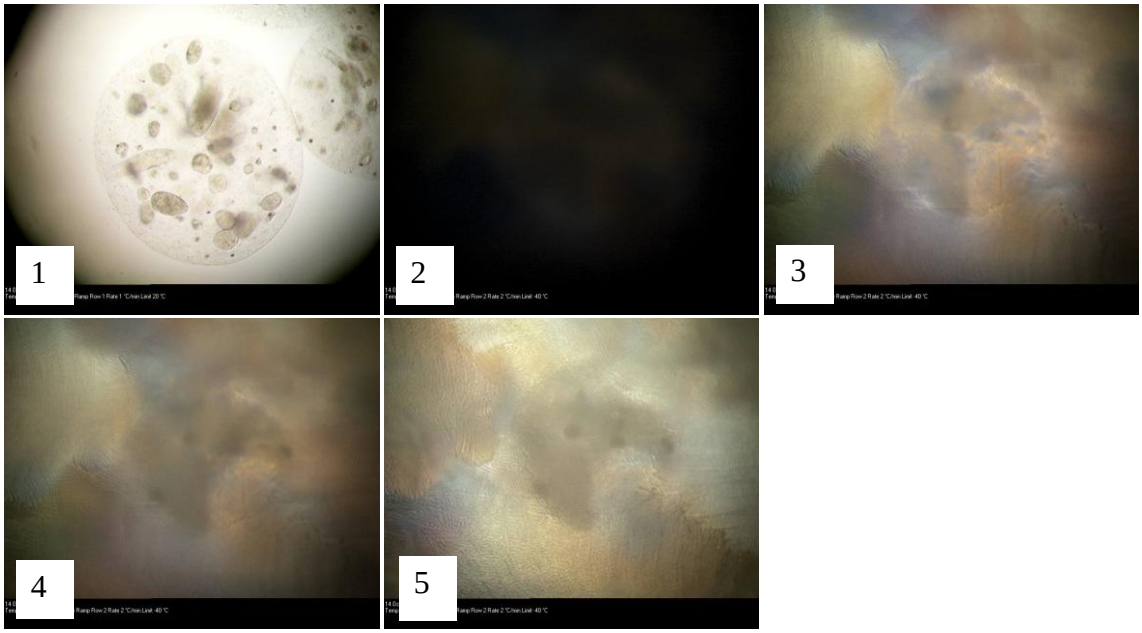


**Figure 3-16. Cryomicroscopy micrographs of ELS cooled with cryoprotectant.**

**Images 1-5 were captured during cooling. Temperatures when images were captured were room temperature, -20°C, -21°C, -22°C and -40°C for images 1, 2, 3, 4 and 5 respectively. Original magnification x4.**

3.4.5.3. Ice formation in presence of DMSO and cholesterol

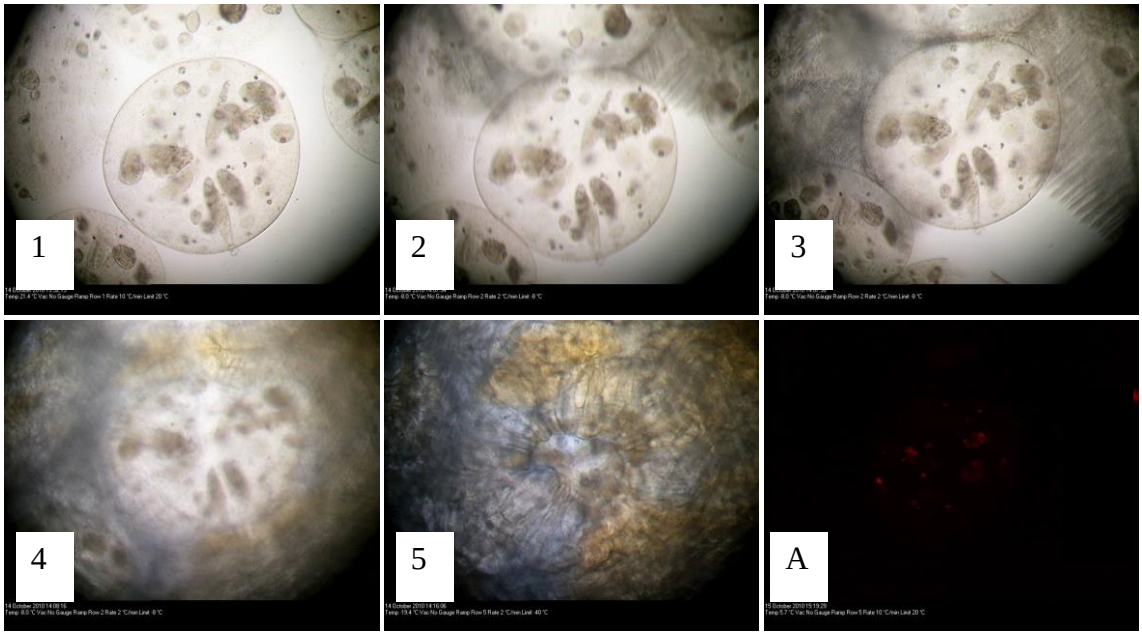


**Figure 3-17. Cryomicroscopy micrographs of ELS cooled with cryoprotectant and cholesterol.**

**Images 1-5 were captured during cooling. Temperatures when images were captured were room temperature, -8°C, -8.5°C, -9°C and -40°C for images 1, 2, 3, 4 and 5 respectively. Image A shows ELS stained with PI and was captured at 20°C post-warming. Original magnification x4.**

When ELS were cooled in DMSO with cholesterol, nucleation occurred at a warmer temperature at -8°C (image 2 in Figure 3-17). Ice formation was also more gradual and ice crystals were observed propagating across the field over approximately 1 minute (images 2-4 in Figure 3-17). Ice formed first around the bead before forming over or through the bead (it was difficult to determine which) and the bead appeared to shrink. However, even by -40°C (image 5 in Figure 3-17), there was no spheroid darkening indicating that intracellular ice formation did not occur. When ELS were stained with PI (image A in Figure 3-17), only very few individual cells stained positive, indicating the membrane integrity had been retained.

### 3.4.6. Effect of minimising supercooling using cholesterol on ELS recovery in post-thaw cultures

When ELS were cryopreserved either with or without cholesterol, large differences in recovery in post-thaw cultures were observed.

3.4.6.1. Effect of cholesterol on viability in post-thaw cultures

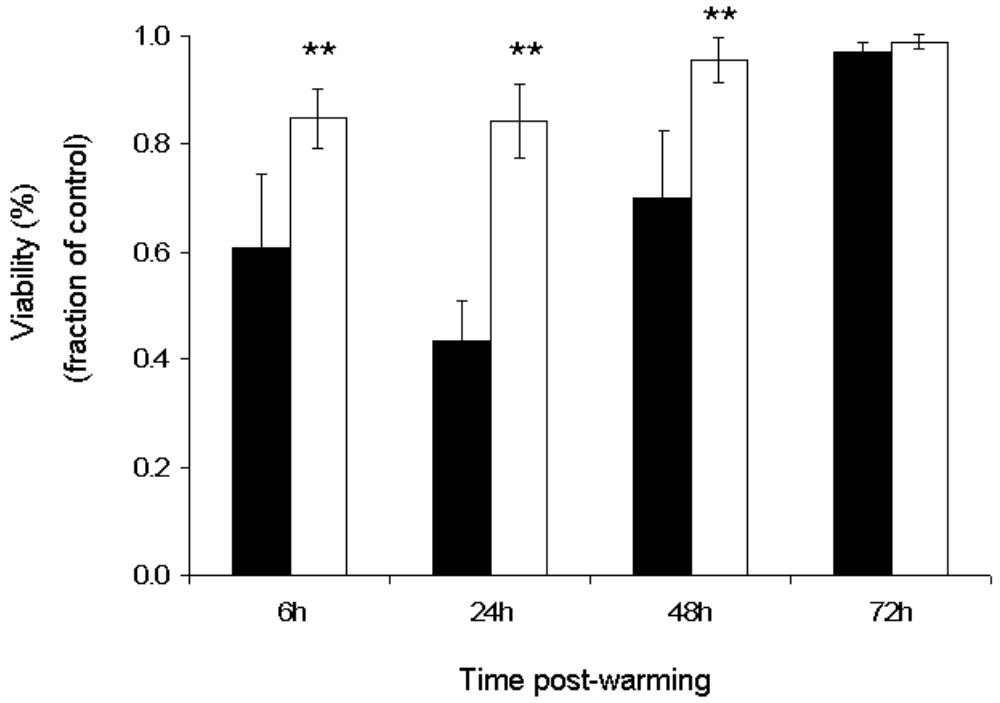


**Figure 3-18. Effect of cholesterol on viability of ELS in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior. ELS were warmed rapidly using a 37°C waterbath. Viability of cryopreserved ELS with or without cholesterol (white or black bars respectively) was quantified, and results expressed as fraction of unfrozen ELS. Values are means of 5 samples from 4 independent experiments +/- SD. **p<0.01. Taken from Massie et al. 2011a.**

Viability was significantly improved at 6, 24 and 48 hours post-warming when cholesterol was included in the CPA media. In the presence of cholesterol, viability in post-thaw cultures was always more than 0.8-fold that of the unfrozen control. By 48 hours post-warming, recovery of ELS cryopreserved with cholesterol was comparable to that of the unfrozen control. By 72 hours, there was no statistical difference between the 2 cryopreserved cohorts as both achieve viability > 0.9-fold of the unfrozen control (Figure 3-18).

3.4.6.2. Effect of cholesterol on viable cell numbers in post-thaw cultures

Viable cell numbers were significantly improved at 24, 48 and 72 hours post-warming when cholesterol was included during cryopreservation. With cholesterol, ELS viable cell numbers were maintained at 0.65-fold that of the unfrozen control over the 72

hours. However, without cholesterol, ELS viable cell numbers were always less than half that of the unfrozen control at 0.3-fold at 24 hours although this did improve to 0.45-fold that of the unfrozen control by 72 hours post-warming.

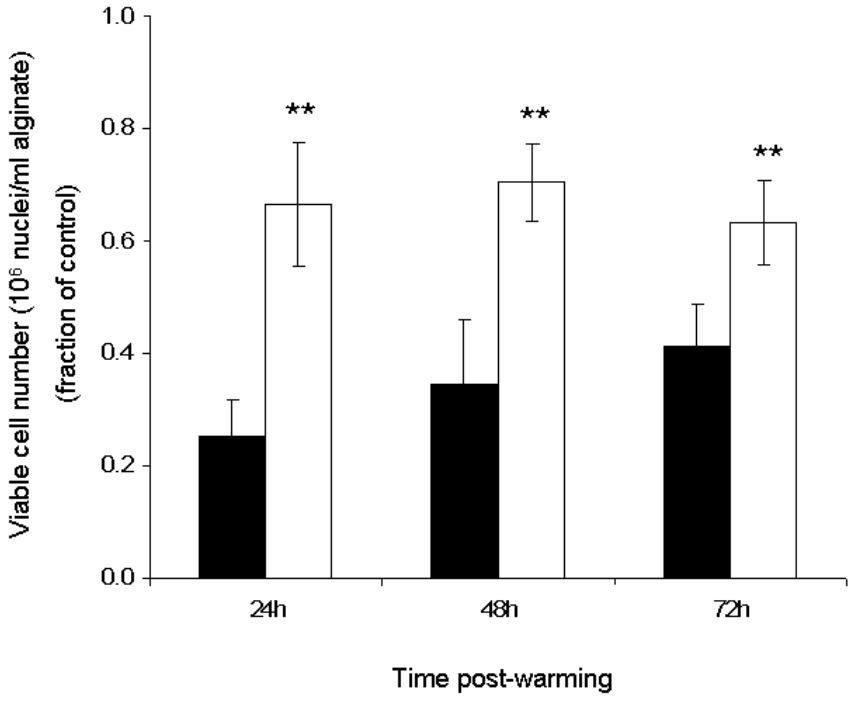


**Figure 3-19. Effect of cholesterol on ELS viable cell numbers in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior. ELS were warmed rapidly using a 37°C waterbath. Viable cell numbers were quantified to 72 hours following cryopreservation and at equivalent time point for unfrozen ELS. Results for cryopreserved ELS with or without cholesterol (white or black bars respectively) are expressed as fraction of unfrozen ELS. Values are means of 5 samples from 4 independent experiments +/- SD. **p<0.01. Taken from Massie et al. 2011a.**

### 3.4.6.3. Effect of cholesterol on function in post-thaw cultures

Functional data is expressed either as total function or normalised to viable cell number or total protein as indicated.

*3.4.6.3.1. Oxidative metabolism*

**Normalised general oxidative metabolism**

MTT reduction was significantly reduced in both cryopreserved cohorts at 24 and 48 hours post-warming compared to the unfrozen control. However, MTT reduction improved in both cryopreserved cohorts progressively and by 72 hours post-warming, there was no difference between all 3 groups. MTT reduction at 24 hours post-warming was approximately doubled when cholesterol was included during cryopreservation but there was no difference between the 2 cryopreserved groups after this.

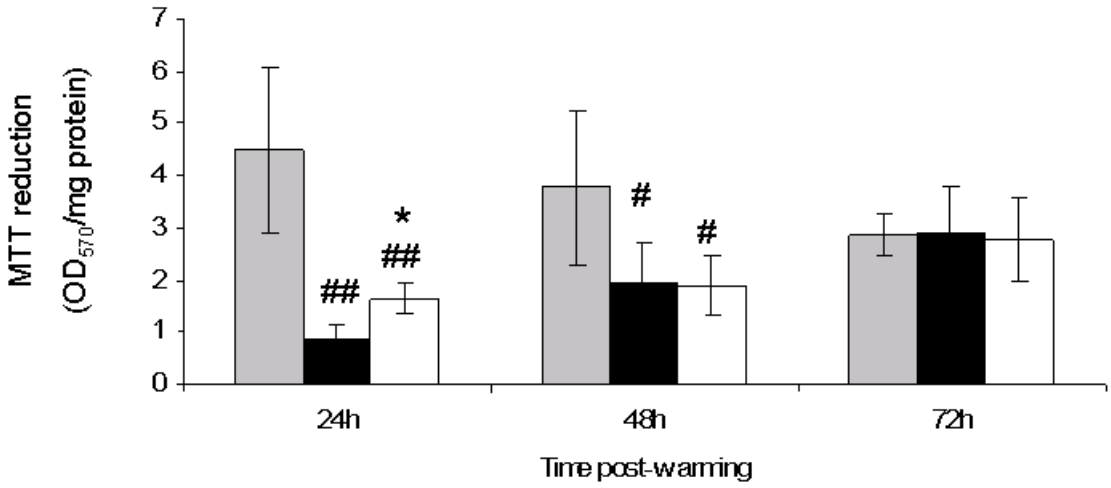


**Figure 3-20. Effect of cholesterol on normalised ELS MTT reduction in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior. ELS were warmed rapidly using a 37°C waterbath. Metabolic activity was assessed using MTT assay. Results for unfrozen ELS (grey), cryopreserved ELS with or without cholesterol (white or black bars respectively) are shown. Values are means of 5 samples +/- SD from a single experiment. *p<0.05 compared to ELS cryopreserved without CholC. #p<0.05, ##p<0.01 compared to unfrozen ELS. Taken from Massie et al. 2011a.**

**Total general oxidative metabolism**

Total MTT reduction was significantly less for ELS cryopreserved without cholesterol compared to unfrozen ELS at all time points measured. When ELS were cryopreserved with cholesterol, this was only true at 24 hours post-warming but thereafter there was no statistical difference between unfrozen ELS and those cryopreserved with cholesterol. However there was a trend that cryopreserved ELS MTT reduction was lower at both later time points. When comparing ELS cryopreserved with and without cholesterol,

ELS cryopreserved with cholesterol showed increased MTT reduction at all 3 time points, significantly so at 24 and 48 hours post-warming.

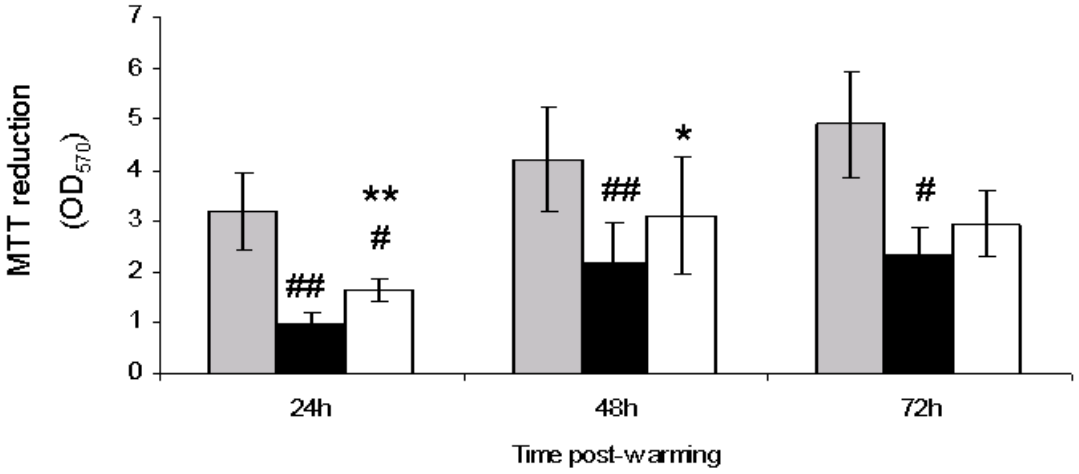


**Figure 3-21. Effect of cholesterol on ELS total MTT reduction in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior. ELS were warmed rapidly using a 37°C waterbath. Results for unfrozen ELS (grey), cryopreserved ELS with or without cholesterol (white or black bars respectively) are shown. Values are means of 5 samples +/- SD from a single experiment. **p<0.01, *p<0.05 compared to ELS cryopreserved without CholC. ##p<0.01, #p<0.05 compared to unfrozen ELS. Taken from Massie et al. 2011a.**

*3.4.6.3.2. Hepato-specific protein synthetic function*

In the following graphs, albumin production is represented as fold change as experiments were repeated. For all other proteins, raw values are shown.

**Normalised synthesis and secretion**

Out to 24 hours post-warming, per cell protein release of all 4 proteins measured (albumin, alpha-1-fetoprotein (AFP), fibrinogen and alpha-1-antitrypsin (AAT)) was higher in ELS cryopreserved without cholesterol compared to ELS cryopreserved with cholesterol. Beyond this, out to 72 hours post-warming, per cell albumin and fibrinogen release was comparable between both cryopreserved groups and the control. Similarly, per cell AFP and AAT production of ELS cryopreserved with cholesterol returned to levels comparable to those of the control by 48h. However, AFP and AAT release of ELS cryopreserved without cholesterol fell over time to ~0.25- and 0.4-fold of control respectively by 72 hours post-warming (Figure 3-22).

**Total synthesis and secretion**

Total albumin production of ELS cryopreserved without cholesterol was ~0.3-fold of control between 24 and 72h but with cholesterol, total albumin production of cryopreserved ELS was improved from 0.3-fold at 24 hours post-warming to ~0.9-fold control by 72h. Similarly, without cholesterol during cryopreservation, AFP secretion was approximately 0.1-fold of control between 24 and 72h but with cholesterol was improved by 48h and maintained to 72h to 0.5-fold of control. Total fibrinogen and AAT secretion were similar for both cryopreserved cohorts at 24h at approximately 0.4-fold of control. Thereafter however, total secretion of both fibrinogen and AAT was improved using cholesterol to approximately 0.6-fold of control, approximately double cf. ELS cryopreserved without cholesterol. To summarise, total synthesis and secretion of all 4 proteins was improved at all time points using INA-modified CPA-medium (Figure 3-23).

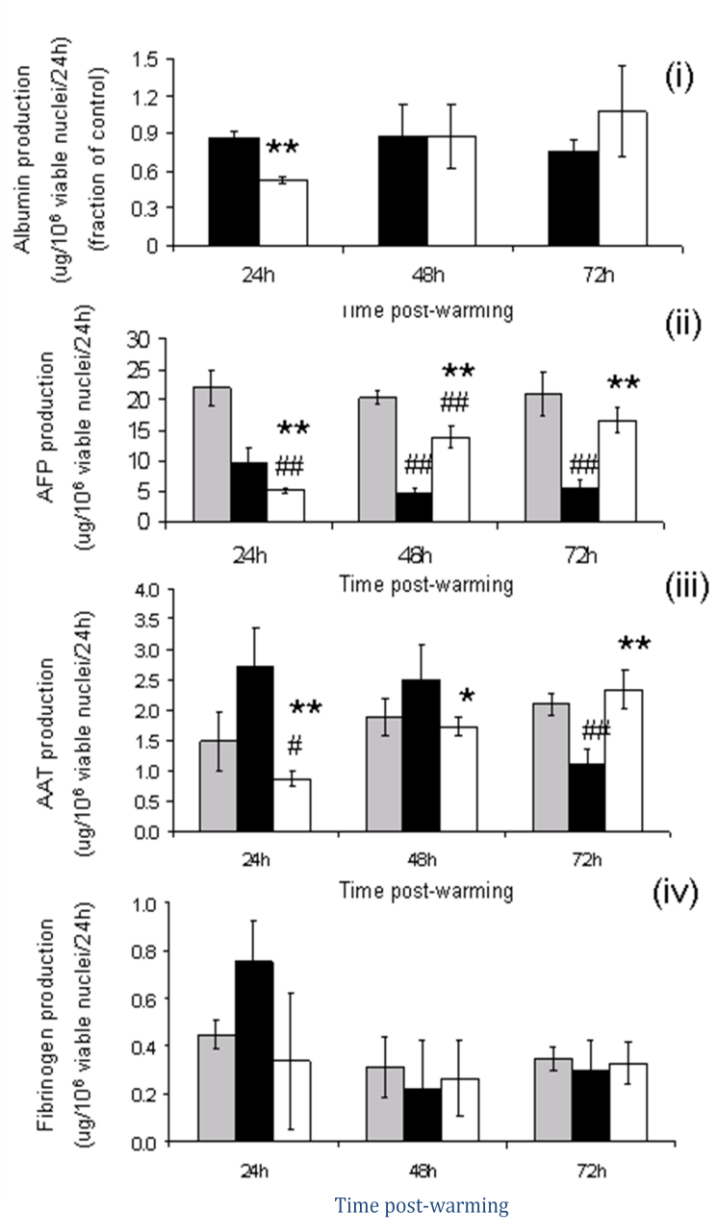


**Figure 3-22. Effect of cholesterol on normalised ELS secretion of hepato-specific proteins in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior and warmed rapidly (37°C waterbath). Albumin (i), AFP (ii), AAT (iii) and fibrinogen (iv) synthesis and secretion over 24 hours was quantified using ELISA to 72 hours following cryopreservation and at equivalent time point for unfrozen ELS. Albumin results for cryopreserved ELS with or without cholesterol (white or black bars respectively) are expressed as fraction of unfrozen ELS and are means of 5 samples from 4 independent experiments +/- SD. AFP, AAT and fibrinogen results for unfrozen ELS (grey), cryopreserved ELS with or without cholesterol (white or black bars respectively) are means of 5 samples +/- SD from a single experiment. **p<0.01, *p<0.05 compared to ELS cryopreserved without cholesterol. ##p<0.01, #p<0.05 compared to unfrozen ELS. Taken from Massie et al. 2011a.**

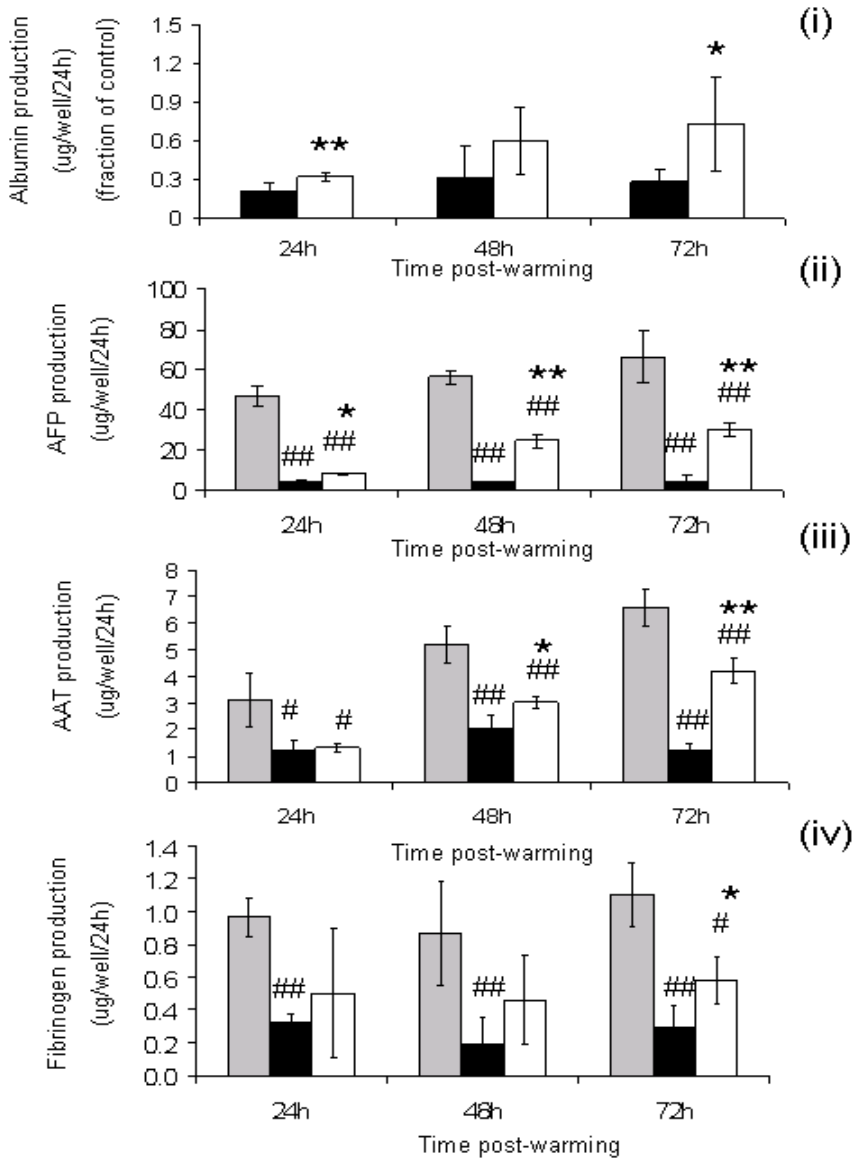


**Figure 3-23. Effect of cholesterol on ELS total secretion of hepato-specific proteins in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior and warmed rapidly (37°C waterbath). Albumin (i), AFP (ii), AAT (iii) and fibrinogen (iv) synthesis and secretion over 24 hours was quantified using ELISA to 72 hours following cryopreservation and at equivalent time point for unfrozen ELS. Albumin results for cryopreserved ELS with or without cholesterol (white or black bars respectively) are expressed as fraction of unfrozen ELS and are means of 5 samples from 4 independent experiments +/- SD. AFP, AAT and fibrinogen results for unfrozen ELS (grey), cryopreserved ELS with or without cholesterol (white or black bars respectively) are means of 5 samples +/- SD from a single experiment. **p<0.01, *p<0.05 compared to ELS cryopreserved without cholesterol. ##p<0.01, #p<0.05 compared to unfrozen ELS. Taken from Massie et al. 2011a.**

*3.4.6.3.3. Cytochrome P450 activity*

**Normalised cytochrome P450 activity**

At 24 hours post-warming, both groups of cryopreserved ELS showed CYP450 function that was approximately one third that of unfrozen ELS. However, both cryopreserved cohorts showed improved function over time and by 48 hours ELS cryopreserved with cholesterol did not differ from the unfrozen control. ELS cryopreserved without cholesterol took longer to recover and, by 48 hours function was significantly lower than both the unfrozen control and also ELS cryopreserved with cholesterol.

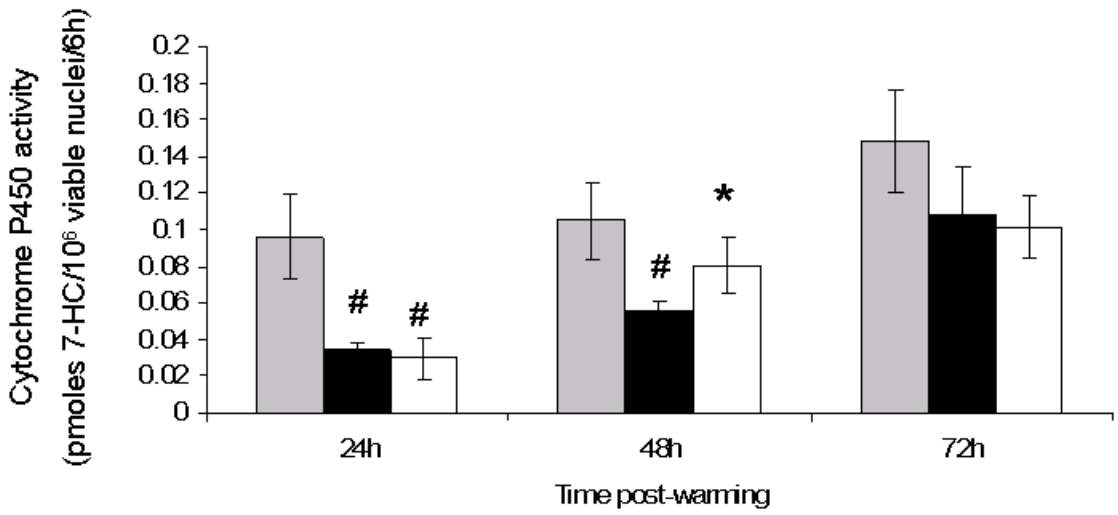


**Figure 3-24. Effect of cholesterol on normalised ELS CYP450 activity in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior and warmed rapidly (37°C waterbath). CYP450 activity was assessed using ECOD assay. Results for unfrozen ELS (grey), cryopreserved ELS with or without cholesterol (white or black bars respectively) are shown. Values are means of 5 samples +/- SD from a single experiment. *p<0.05 compared to ELS cryopreserved without cholesterol. #p<0.05 compared to unfrozen ELS. Taken from Massie et al. 2011a.**

**Total cytochrome P450 activity**

At 24 hours post-warming, both cryopreserved cohorts showed significantly reduced CYP450 activity compared to unfrozen ELS. ELS cryopreserved with cholesterol showed approximately twice the activity of ELS cryopreserved without cholesterol although this was not a statistically significant improvement. At 48 hours post-

warming, ELS cryopreserved without cholesterol did not improve whereas ELS cryopreserved with cholesterol improved by approximately 2-fold, significantly cf. ELS cryopreserved without cholesterol ($p<0.01$). By 72 hours, both cohorts had improved further but ELS cryopreserved with cholesterol maintained a statistically significant advantage over those ELS cryopreserved without cholesterol.

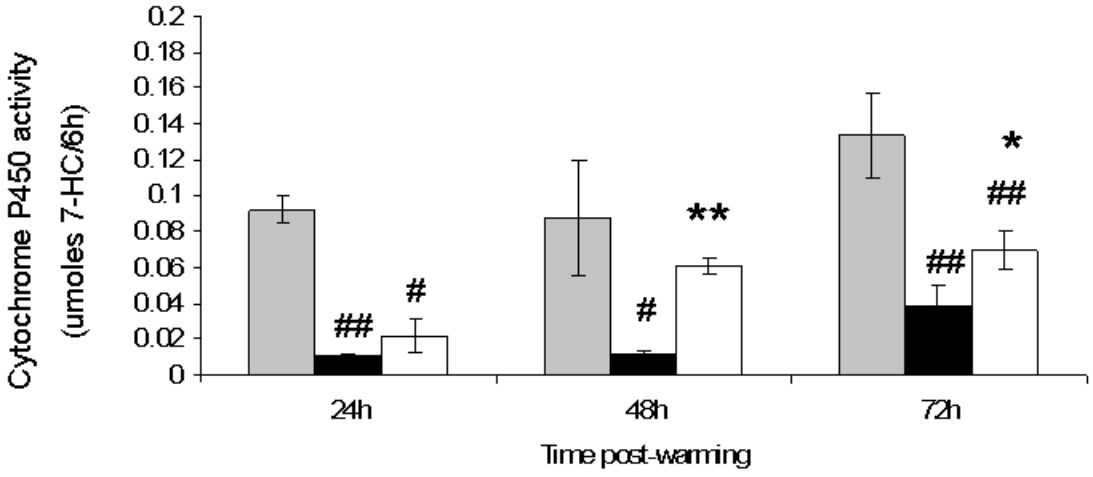


**Figure 3-25. Effect of cholesterol on total ELS CYP450 activity in post-warming cultures.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/Celsior and warmed rapidly (37°C waterbath). CYP450 activity was assessed using the ECOD assay. Results for unfrozen ELS (grey), cryopreserved ELS with or without cholesterol (white or black bars respectively) are shown. Values are means of 5 samples +/- SD from a single experiment. **p<0.01, *p<0.05 compared to ELS cryopreserved without cholesterol. ##p<0.01, #p<0.05 compared to unfrozen ELS. Taken from Massie et al. 2011a.**

### 3.4.7. Warming rates

ELS were warmed using different protocols to achieve different warming rates and recovery of ELS assessed in post-thaw cultures

3.4.7.1. Temperature profiles

ELS were thawed most rapidly using 37°C water which took approximately 120 seconds. ELS thawed in 20°C water took approximately 30 seconds longer. ELS thawed in air at 20°C took considerably longer, approximately 900 seconds. ELS

thawed at 4°C in air took longest at nearly 1500 seconds. For all 4 protocols, the warming rate decreased as sample temperature increased (Figure 3-26).

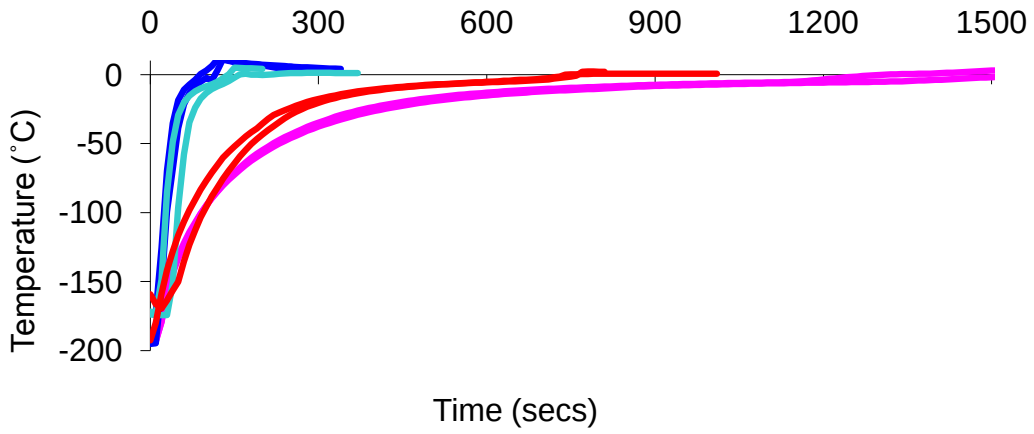


**Figure 3-26. Temperature profiles during warming.**

**ELS were warmed in either 4°C air (pink), 20°C air (red), 20°C water (light blue) or 37°C water (dark blue) after storage in nitrogen. Two samples were thawed using each protocol (each indicated by a single line) within the same experiment.**

3.4.7.2. Effect of warming rate on ELS recovery in post-warming cultures

Optimal recovery of ELS was found when rapid warming rates (using 37°C water) were applied.

*3.4.7.2.1. Viability*

Viability in all 4 cryopreserved groups was significantly reduced compared to the unfrozen control. The greatest loss of viability was seen in ELS warmed using 4°C air and was approximately 10%. At warming rates greater than this, there was a positive correlation between warming rate and recovery with ELS warmed most rapidly in 37°C water showing only a small (although significant) drop of approximately 4% viability (Figure 3-27).

*3.4.7.2.2. Viable cell number*

Viable cell numbers of all 4 cryopreserved groups of ELS were significantly reduced compared to the unfrozen control. Viable cell numbers of ELS warmed in air were slightly less than half that of the unfrozen control. ELS warmed in 20°C air showed slightly better recovery with viable cell numbers at approximately half that of the unfrozen control. ELS thawed in water, either 20°C or 37°C, showed significantly

improved recovery compared to those thawed in air at approximately 80% that of the unfrozen control (Figure 3-28).

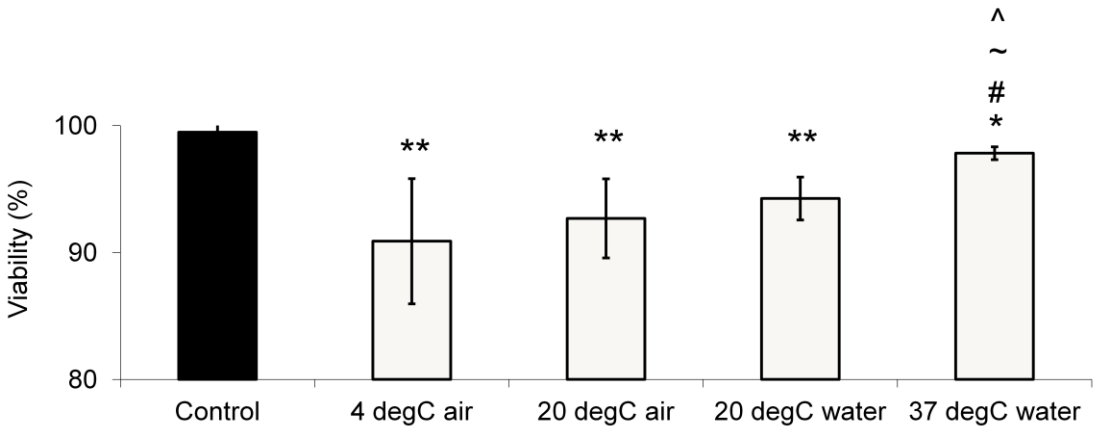


**Figure 3-27. Effect of warming rate on ELS viability.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW before being warmed using different protocols as indicated on the x axis to achieve different warming rates. Viability of cryopreserved ELS was compared 24 hours post-warming and compared to unfrozen (control) ELS at equivalent time point. Data are means of n=5 +/- SD from a single experiment. *p<0.05, **p<0.01 cf. control, #p<0.05 cf. 4°C air, ~p<0.05 cf. 20°C air, ^p<0.05 cf. 20°C water.**

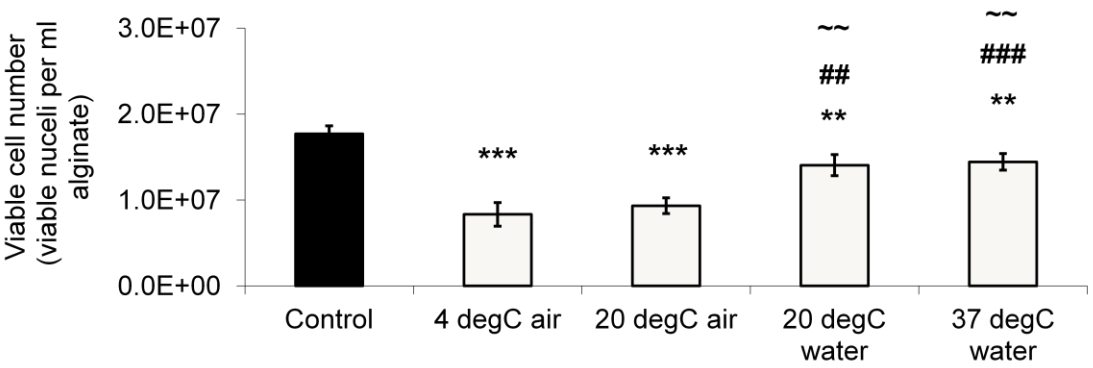


**Figure 3-28. Effect of warming rate on ELS viable cell numbers.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW before being warmed using different protocols as indicated on the x axis to achieve different warming rates. Viable cell numbers of cryopreserved ELS was compared at 24 hours post-warming and compared to unfrozen (control) ELS at equivalent time point. Data are means of n=5 +/- SD from a single experiment. **p<0.01, ***p<0.005 cf. control, ##p<0.01, ###p<0.005 cf. 4°C air, ~~p<0.01 cf. 20°C air.**

*3.4.7.2.3. Function*

ELS total protein synthesis and secretion was significantly reduced in all cryopreserved groups compared to the unfrozen control. Again, this was most apparent for ELS warmed slowly in 4°C air where total protein production was approximately one third that of the unfrozen control. At more rapid warming rates, protein production was increased and when ELS were warmed in 37°C water, total protein production was nearly two thirds that of the unfrozen control, twice that of ELS warmed in 4°C air.

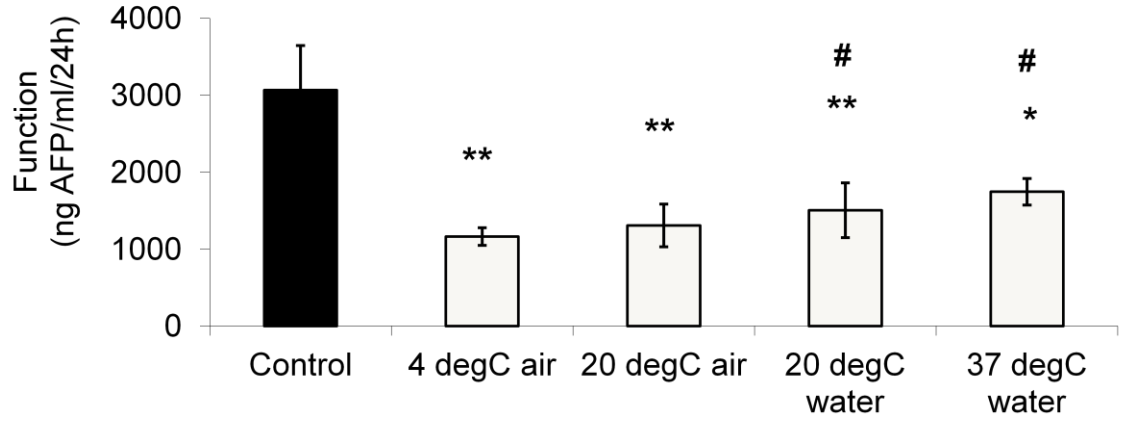


**Figure 3-29. Effect of warming rate on ELS function.**

**ELS were cooled using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW before being were warmed using different protocols as indicated on the x axis to achieve different warming rates. Function of cryopreserved ELS was compared at 24 hours post-warming and compared to unfrozen (control) ELS at equivalent time point. Data are means of n=5 +/- SD from a single experiment. **p<0.01, *p<0.05 cf. control, #p<0.05 cf. 4°C air.**

## 3.5. Discussion

The aims of this Chapter were to characterise ELS recovery in post-thaw cultures and also to determine the optimal cooling and warming rates for ELS. Whilst characterisation of cell recovery following cryopreservation is always useful and of interest, here, it is vital. ELS are intended for use within a clinical setting for treatment of acute liver failure, where treatment should commence soon after diagnosis, so a rapid (i.e. within 48 hours) recovery is essential to the success of any BAL treatment. One of the most fundamental aspects of cryopreservation success is the selection and application of cooling rate. At sub-optimal cooling rates, cells are likely to suffer the effects of solution effects injury. Conversely, at supra-optimal cooling rates, the likelihood of lethal IIF is increased. Similarly, the correct warming rate should be determined to avoid damaging recrystallisation and/or devitrification events (Fuller et al. 2004).

To determine the optimal cooling rate, ELS were cooled at a range of rates between 2 and 30°C/minute using a controlled rate freezer or more rapidly by immersion into liquid nitrogen. When ELS were stained with FDA and PI, large differences were apparent. At all cooling rates greater than 2°C/minute, high levels of background FDA staining and PI staining were apparent. PI stains nuclei of cells where membrane-integrity has been lost. The PI staining results indicate that at cooling rates greater than 2°C/minute, viability of ELS has been lost. Despite this, FDA staining was still seen in these ELS exposed to the faster cooling rates, albeit at a lower intensity and not always localised to ELS. This was an unexpected result but may occur after FDA is cleaved to form its fluorescent product by esterases that persist in cytoplasm of (sub-lethally) damaged ELS. Thereafter, as ELS membranes have been damaged, which is apparent from positive-PI staining, the fluorescent FDA product can then exit ELS to produce an abnormally high background fluorescence.

When ELS were cryopreserved using a narrower range of cooling rates (1, 2 or 3°C/minute) little difference was apparent between the 3 cohorts, although by 72 hours post-warming, 2°C/minute showed optimal recovery. 2°C/minute or similar, has been previously described as the optimal cooling rate for liver cells (Diener et al. 1993; Terry et al. 2006) in agreement with the results obtained here.

Having determined the optimal cooling rate for ELS, recovery was characterised over the clinically-relevant time period (i.e. out to 48 hours post-warming and beyond). When viability of ELS was measured immediately post-warming, only a few cells stained positive for PI, indicating that only few cells had lost membrane integrity. However, out to 6 hours and continuing to 24 hours, incidence of PI staining increased indicating a progressive loss of cell viability. This has previously been observed in other cells (kidney cells) and has been termed cryopreservation-induced-delayed-onset-cell-death (CIDOCD). In that study, kidney cells were cryopreserved in a range of solutions and numbers of intact cells measured in post-thaw cultures out to 48 hours. Recovery between the cryopreserved cells did differ but similar trends in recovery were observed for all cohorts. By between 6 and 24 hours, a "nadir" in cell survival was seen. This was attributed initially to necrosis (at 6 hours post-warming) followed by apoptosis (at 12 hours post-warming) (Baust et al. 2001). This timescale is similar to that observed for ELS in this study.

When ELS were assessed in post-thaw cultures out to 72 hours, a "recovery period" was observed where levels of PI staining decreased relative to FDA staining, resulting in improved viability. There are 3 ways in which this result could arise. Firstly, it is possible and probable, that ELS surviving the cryopreservation process continue to proliferate increasing the amount of FDA staining. Alternatively, PI staining could be decreased as ELS regain membrane integrity as repair processes are initiated in sub-lethally damaged ELS. Such repair processes have been previously described in rat hepatocytes and occur between 24 and 48 hours post-warming (Watts & Grant 1996). This time period is highly comparable to that observed in ELS. Finally, lethally damaged ELS will degenerate and no longer exist to participate in PI staining. In LG BAL alginate beads, HepG2 cells will remain entrapped within the alginate polymeric matrix and so these PI-positive cells may persist longer than if HepG2 cells were cultured as monolayer. In monolayer cultures, non-viable cells would become unattached and would be washed away by medium changes.

The viability results shown here promote the concept that viability is not a static property and can vary with time. This phenomenon also supports the idea of using more than one method of assessment for ELS recovery. As a result, ELS recovery was normally measured by looking at cell numbers and function in addition to viability to give a holistic view of ELS recovery in post-thaw cultures.

A side point for consideration is that cryopreservation of hepatocytes has often been attempted and reported success varies dramatically despite the use of broadly similar cryopreservation protocols. These variations may arise from either assay choice (i.e. membrane integrity assays tend to over-estimate recovery compared to more complex functional or attachment assays) or from the time in post-thaw culture that recovery was measured (i.e. recovery may be over-estimated if assessed before CIDOCD is expressed or after initiation of repair processes).

The large decline in viability observed at 24 hours post-warming of greater than 50% is unacceptable for ELS to be used as the biomass of a BAL despite the selection of the slow cooling rate. Although this cooling rate was applied by the CRF as intended, when sample temperatures were measured during cooling, it became apparent that significant supercooling was occurring. Supercooling is known to be problematic and will increase the likelihood of intracellular ice formation. When nucleation occurs, ice will form extracellularly and the latent heat of crystallisation will be released which increases the temperature of the sample within the cryovial. Meanwhile, the chamber temperature will continue to fall and a large difference in temperature between the sample and chamber will arise. The sample temperature will then fall more rapidly than intended. This in turn increases the likelihood of IIF (Kleinhans 1998).

There are 3 commonly employed methods that are used to limit supercooling. The most common of these is "seeding" where nitrogen-cooled forceps are touched to the external side of cryovial or straw which results in localised deep supercooling of the sample. Where this localised deep supercooling is applied, nucleation is more likely to occur, and so nucleation is initiated within the rest of the sample at temperatures closer to the equilibrium melting point (i.e. less supercooling occurs throughout the majority of the sample) (Whittingham 1977). Another approach, and one that was applied here (albeit largely ineffectively), with the same mechanism is the use of a thermal shocking cooling profile whereby the edge of the sample is cooled but by the chamber temperature as opposed to nitrogen-cooled forceps. This approach has been described as sufficient for cryopreservation of single cell suspensions of hepatocytes (Diener et al. 1993; Terry et al. 2006). Neither of these approaches though are necessarily suitable for use within cryopreservation protocols where large volumes are required as it is difficult to remove sufficient heat from large volumes, even locally.

The third method is that of heterogeneous nucleators. Homogeneous nucleation of water occurs at approximately -40°C and so any nucleation events above this temperature are considered to be heterogeneous, initiated by foreign particles or material defects on the inside of cryovials for example. Heterogeneous nucleators are materials that possess similar crystal lattice size and type to that of ice and act by ordering water into an ice-like conformation. From this initial seed, nucleation can propagate throughout the remainder of the sample to reduce supercooling (Gobinathan & Ramasamy 1983; Head 1961; Rasmussen et al. 1975).

Heterogeneous nucleators are well-described in the literature and have been utilised in cryopreservation protocols previously. Microbial proteins are known to act as heterogeneous nucleators but were not considered suitable for use here where treatment of human patients is the intended end use (Missous et al. 2007). Silver iodide has also been utilised previously (Kojima et al. 1988) and when tested here, did reduce supercooling significantly although not as effectively as cholesterol.

Cholesterol, along with other sterols, has also been previously recognised as an effective heterogeneous nucleator (Head 1961) but has not previously been used within cryopreservation protocols of hepatocytes or spheroids. Demonstrably, cholesterol was an effective heterogeneous nucleator, capable of reducing supercooling by around 11°C as seen by analysis of the time-temperature profiles. In theory, one would expect this to reduce the likelihood of IIF.

Cryomicroscopy was used to observe ELS during cooling, including the nucleation event, and subsequent ice formation and propagation. To establish how IIF is manifested, ELS were first cooled in the absence of DMSO. The observed field darkened as supercooled water nucleated to form ice. This darkening has been described previously and results from scattering of light by newly-formed ice crystals (Grout & Morris 1987; Leibo et al. 1978). IIF was also seen at a slightly lower temperature and, again, was characterised by darkening. Ice propagation has been demonstrated in several cell types such as salivary gland cells (Berger & Uhrik 1996) and kidney cells (Acker et al. 1999) but also in HepG2 cells (Irimia & Karlsson 2005). As a result, inter-connected cell types are more likely to undergo IIF at warmer temperatures than single cells and this has been demonstrated experimentally using hamster fibroblast cells where 50% of single cells contained ice at -9.4°C compared to

-6.6°C for cell spheroids (Acker et al. 1999). Indeed, here fluorescent microscopy with PI revealed total loss of membrane integrity with all spheroids staining positive for PI.

It should be pointed out that I was using the rapid darkening of the different spheroids within the ELS as a surrogate for IIF, and at this magnification, it is not possible to speak objectively about intracellular ice forming in individual cells. However, the rapid darkening event has been widely accepted as a consequence of IIF in different cell systems over many years (Leibo et al. 1978). In ELS, there was a temporal separation between the extracellular ice nucleation and the 'black flashing' of the spheroids, indicating that the IIF formed as a result of the presence of extracellular ice. Exactly how the two events are linked remains to be determined conclusively but it has been suggested that the formation of extracellular ice alters the topography of the internal cell membrane, resulting in formation of nucleation sites that permit IIF (Toner et al. 1993).

When ELS were cooled with DMSO but without cholesterol, nucleation occurred at a lower temperature than previously observed but supercooling was still apparent: ice nucleation was well below the equilibrium melting point and propagated rapidly. Again, some IIF was apparent but at a lower temperature than without DMSO and was not present within all ELS. This result is expected as DMSO is known to lower the nucleation point by dispersing water molecules and reduces the likelihood of IIF by not only depressing the nucleation temperature but also simultaneously allowing more time for cell dehydration by exosmosis (Fuller et al. 2004).

When ELS were cooled with DMSO and cholesterol, nucleation occurred at higher temperatures providing visual confirmation of time-temperature profiles. Nucleation was also much less sudden and in fact took approximately 1 minute to propagate over the observed field. As ice propagation was slower it was possible to observe that ice did not grow through the alginate bead and it appeared that the presence of the alginate (despite being a hydrogel and, therefore, >99% water) interrupted ice crystal growth. This has been observed previously although in the study by Kusano et al, the alginate beads were coated with poly-L-lysine (unlike ELS beads) (Kusano et al. 2008). As the alginate provided a temporary barrier to ice growth, this would allow more time for exosmosis to occur, decreasing the likelihood of IIF and indeed, there was no evidence of IIF.

Exosmosis can normally be seen as cell shrinking as the cell dehydrates its mobile water fraction. In single cells this can be clearly observed in some instances. Here, it is

difficult to see this for a number of reasons. Firstly, the alginate beads captured here are relatively large (~450μm diameter) and the resolution of images is sub-optimal due to the depth of field. In addition, it is difficult to view individual cells as cell-cell contacts join individual cells together to form spheroids. It may therefore be possible to observe shrinkage of entire spheroids.

However, as ELS produce large amounts of extracellular matrix (ECM) which could possibly mask cell shrinkage, it is difficult to observe. Also, spheroids are not spherical, but instead are typically elliptical, and it is difficult to measure shrinkage as reference points on individual spheroids should be used to take these measurements which is complicated by the irregular shape of the spheroids. Finally, during cooling following nucleation, as the ice front advanced around the alginate beads, the beads were rolled slightly in a random direction which again makes measurements of cellular dehydration difficult. No studies investigating cryo-dehydration of cell spheroids or tissues following ice formation could be found and so it is difficult to make comparisons to the results observed here although cryo-dehydration of inter-connected cell types (notably pancreatic islets) has been observed in response to exposure to hypertonic solutions at sub-zero temperatures (de Freitas et al. 1997).

An unexpected observation was the shrinkage of the entire alginate bead presumably in response to increasing concentrations of excluded solutes following ice formation outside the alginate beads. The structure of the alginate bead was considered to be uniform (i.e. a continuous heterogeneous polymer matrix) but the results from cryomicroscopy suggest otherwise. The alginate appeared to interrupt the progression of the ice front and this coupled with the observed bead dehydration seems to suggest that a "crust" is present around the edge of the bead that acts in a similar way to the cell membrane. This has the effect of allowing more time for ELS dehydration to occur and this along with the use of cholesterol are the probable mechanisms of protection for ELS cryopreserved in this method.

Alginate encapsulation/sandwich has previously been demonstrated to protect other cell types (neurons) (Malpique et al. 2010) but also hepatocytes (Kusano et al. 2008; Mahler et al. 2003; Rialland et al. 2000) during cryopreservation. The mechanism of protection appears to be that alginate slows ice formation allowing more time for cellular dehydration and provides a support matrix for cells to adhere to. This is most apparent for sandwich configurations where in the absence of alginate, adhered cells are torn

from the matrix as ice forms whereas in sandwich configuration, cells may be protected (Giri et al. 2010).

Having established that cholesterol is an effective ice nucleator (from time-temperature profiles) and that its inclusion during cryopreservation results in reduced incidence of IIF allowing maintenance of membrane integrity (from light and fluorescent cryomicroscopy), its efficacy in improving ELS recovery after cryopreservation was assessed. ELS recovery was measured out to 72 hours post-warming and in order to gain a holistic overview of ELS recovery, viability, cell number and three functional assays were utilised.

As for ELS cryopreserved with cholesterol, the minimum viability was seen at 24 hours post-warming but the loss in viability was small compared to those cryopreserved without cholesterol. At 24 hours, ELS cryopreserved with cholesterol showed a 40% improvement in viability cf. those cryopreserved without cholesterol. This advantage was also true at 6 and 48 hours post-warming. However, by 72 hours, both cryopreserved cohorts showed similar recovery. Despite the recovery of ELS cryopreserved without cholesterol, viable cell numbers were still significantly higher at all time points when cholesterol was present during cryopreservation. This is important as ELS comprise the biomass for the BAL and large cell numbers will be required to treat an adult patient suffering from ALF.

Function of ELS was also assessed in post-thaw cultures. Results were expressed as total function or normalised to cell number or protein. MTT reduction which is a measure of general oxidative metabolism was reduced in both cryopreserved cohorts compared to unfrozen ELS but was greater when cholesterol was included. These differences were most apparent at 24 hours post-warming before a recovery period, in line with viability data, indicating that the fluorescent staining methodology used to quantify viability in ELS is robust. However, MTT reduction is not a measure of hepato-specific function and so CYP450 function and protein synthesis and secretion were also measured.

Total CYP450 function was also reduced in both cryopreserved cohorts compared to unfrozen ELS. No information regarding the loss of CYP450 function after cryopreservation in HepG2 cells could be found for either single cells or spheroids but the results here align well with data from rat hepatocytes (Grondin et al. 2008). ELS cryopreserved with cholesterol showed improved total function at all time points

measured. When CYP450 function was normalised to cell number, there was no difference between the cryopreserved cohorts at 24 hours but by 48 hours, those cryopreserved with cholesterol showed increased function, indicating a more rapid recovery.

For the CYP450 assays, ELS were induced using indirubin. CYP450 function in HepG2 cells is known to be low but can be increased using chemical induction (Westerink & Schoonen 2007) (such as with indirubin) to ensure results can be reliably quantified. Although induction was deemed necessary, it does present an additional factor when interpreting these results as ELS with very different viabilities (up to 60% between unfrozen and unfrozen without cholesterol, for example) may possess different capabilities to respond to induction. Although a previous study using human hepatocytes where an, admittedly smaller, loss of 20% viability was seen, did not affect inducibility (Silva et al. 1999).

Alternatively, CYP450 function can be increased in 3D cultures cf. monolayer cultures (Khalil et al. 2001) and so maintenance of the 3D structure is likely to be required to maintain this upregulated function. Demonstrably, the use of cholesterol, reduces the incidence of IIF which clearly does improve both viability, cell number and function but inter-cellular ice formation may continue to be problematic for ELS. If inter-cellular ice forms, it may well interrupt the 3D structure of ELS to result in decreased function.

This mode of injury also likely applies to synthetic function of ELS. Synthetic function of ELS was assessed from synthesis and secretion of 4 proteins: albumin, AFP, AAT and fibrinogen. Albumin is of great importance for any BAL as it is required to maintain oncotic pressure and transporter function, and is also a good marker for ELS functionality as it approaches that of human hepatocytes (unlike CYP450 which is far lower) (Khalil et al. 2001). AFP is not produced by adult hepatocytes but again is a good indicator of ELS functionality as ELS produce AFP in similar quantities to albumin. AAT and fibrinogen are produced in smaller amounts but again, provide a means of a more complete assessment of recovery.

Normalised synthetic function of ELS cryopreserved with cholesterol was comparable to that of unfrozen ELS by 72 hours post-warming. At the earlier time points, per cell production was lower for all 4 proteins, most notably at 24 hours post-warming. This again suggests that a recovery period is initiated beyond 24 hours, which is relevant for

BAL treatment. At all time points, total production of all 4 proteins was greater for ELS cryopreserved with cholesterol cf. those cryopreserved without cholesterol.

Interestingly, at 24 hours, per cell production of all 4 proteins was greater in ELS cryopreserved without cholesterol than with. This is a surprising result but may be caused by unregulated release of proteins that were synthesised before cryopreservation that leak from membrane-damaged ELS in post-thaw cultures. This is most apparent for A1AT and fibrinogen which are produced in lower quantities than AFP and albumin and so this unregulated release is large compared to *de novo* synthesis. This result also shows that sustained recovery of function must be demonstrated to guarantee efficacy of treatment when using cryopreserved ELS.

Of the assays to measure function in ELS, only synthesis and secretion of the 4 proteins and detoxification of 7-EC provide a means of assessing liver-specific function. Measuring liver-specific function is important not only because it determines the success of any cryopreservation protocol applied but also because it is what ultimately determines whether or not a BAL utilising ELS will be clinically useful. As cytochrome P450 activity is known to be considerably lower in HepG2 cells than primary human hepatocytes, even with induction, it is difficult and expensive to measure. Additionally, as ELS require induction this complicates interpretation of results as it is unknown how cryopreservation affects inducibility of ELS.

Conversely, hepato-specific protein production from ELS is easy to measure, does not require addition of any components to culture medium and, at least for albumin, is comparable to that of primary human hepatocytes and will therefore, be used to assess function for the remainder of this thesis. As the per cell production of all 4 proteins yielded an unexpected result (in that it was higher in ELS cryopreserved without cholesterol than with) at 24 hours post-warming and was not sustained thereafter, although interesting, these results are not truly representative of ELS recovery. Moreover, in terms of BAL efficacy, per cell functionality is not necessarily relevant. Ultimately, any cryopreservation protocol should aim to provide the same total amount of function as the fresh equivalent. For these reasons, total protein production will be used to determine efficacy of applied cryopreservation protocols in this thesis.

To summarise the effect of including cholesterol during cryopreservation, cholesterol improves recovery of ELS in post-thaw cultures as assessed from viability, cell number and, crucially, functionality. These results were dramatic and although the effects of

supercooling are known to be damaging for single cells, the effects are devastating for ELS. Strategies to avoid IIF in the first instance include the selection of a sufficiently slow cooling rate without exposing cells to solution effects injury and ensuring that supercooling is avoided, in this study achieved using cholesterol.

HepG2 cells within ELS are inter-connected proven by using transmission electron microscopy (Selden et al. 1999). The notion of ice propagation between inter-connected cells is not a new one and has been demonstrated experimentally in other cell types (Acker et al. 1999; Berger & Uhrik 1996). For ELS, this means that once ice is present in any cell within a spheroid it may propagate throughout the entire spheroid, rendering that spheroid non-viable.

The likelihood of IIF in the first instance is also increased in ELS cf. single cells as water transport characteristics make freezable water more likely to persist in ELS. More time is required for dehydration of ELS as water must exit cells sequentially from cells in the centre of spheroid or aggregate to cells on the edge of the spheroid via aquaporins before exiting the spheroid in response to the osmotic gradient (Ehrhart et al. 2009; Korniski et al. 1999). This makes ELS susceptible to IIF. The non-linear cooling profile used here was sufficient to avoid IIF in single cell suspensions of hepatocytes and although successfully applied here, was insufficient to avoid damaging IIF in ELS which highlights the challenge of cryopreserving inter-connecting cell types.

As well as selecting and applying the optimal cooling rate, the optimal warming rate should also be determined. For cells cooled using rapid cooling rates, where ice crystals are small, rapid warming rates are generally considered to result in the best recovery. This is because rapid warming rates avoid recrystallisation of small ice crystals to larger, more thermodynamically stable, crystals. Recrystallisation can mechanically disrupt cell membranes and organelles and cell-cell contact and tissue structure to result in decreased recovery in post-thaw cultures. For slowly cooled cells, it remains unclear whether rapid or slow warming rates should be applied but in this study, rapid warming rates appear to be optimal. This in agreement with findings from others (Terry et al. 2006; Terry et al. 2010) although the reasons for rapid warming rates resulting in improved recovery are unclear. However, rapid warming rates do reduce the length of exposure to high concentrations of CPA during warming as CPA can be washed out sooner.

Although significant improvements to the cryopreservation protocol have been achieved, recovery of cryopreserved ELS is still insufficient to be used within a BAL. As described earlier, CIDOCD is likely to result from both apoptosis and necrosis. Necrosis may be caused by IIF and cholesterol has been demonstrated to reduce this. Apoptosis, on the other hand, is likely to be caused by abnormal cell signalling in response to the stresses encountered during cryopreservation and so methods to limit this apoptotic response will be investigated in the next Chapter.

## 3.6. Conclusions

Encapsulated liver cell spheroids (ELS) suffer from cryopreservation-induced-delayed-onset-cell-death which is most apparent 24 hours post-warming and results in a significant loss in cell viability.

ELS are optimally cryopreserved using slow cooling rates in the region of 2°C/minute and supercooling must be avoided.

Supercooling is apparent from time-temperature profiles where large releases in latent heat of crystallisation can be observed. Supercooling is best avoided using cholesterol which results in improved viability, cell number and function.

ELS are particularly prone to intracellular ice formation as they are inter-connected which makes cellular dehydration more difficult and ice is able to propagate through spheroids via cell-cell contacts.

Rapid warming rates offer optimal recovery and should be used to thaw ELS.

Despite the protocol improvements instigated in this Chapter, ELS recovery is still insufficient for a BAL and in the next Chapter, methods to reduce apoptosis will be investigated.

# CHAPTER 4

# Cryopreservation Stresses

## 4.1. Introduction

Having characterised encapsulated liver cell spheroid (ELS) recovery following cryopreservation in the previous Chapter, it is clear that cryopreservation-induced-delayed-onset-cell-death (CIDOCD) occurs. The aim of this Chapter is to limit CIDOCD. CIDOCD has been observed in other cell types following cryopreservation and has been attributed to both apoptosis and necrosis (Baust et al. 2000).

### 4.1.1. Cell death

For a bioartificial liver (BAL), any cell death will impact negatively. Firstly, ELS cell death will reduce the biomass available which means that the BAL will be less effective for treatment of acute liver failure (ALF). In addition to this, cell death presents the possibility of cellular content, including DNA, returning to the patient. Any cellular material may trigger an immune or inflammatory response from the host. Furthermore, inadvertent release of HepG2 DNA release back to the patient also poses a potential risk of tumourigenesis as HepG2 cells are derived from a hepato-blastoma. In the final BAL, a filter system will be in place as part of the circuit to limit/prevent this, but by reducing cell death following cryopreservation, the load on these filters and the risk to the patient is reduced.

#### 4.1.1.1. Necrosis

In the previous chapter, cholesterol was identified as an effective heterogeneous nucleator that reduced IIF and membrane damage, but some cell death was still evident and function remained low, before initiation of recovery period, indicating that there was potential for further improvement. Necrotic cell death is uncontrolled and results from external influences (i.e. is not a result of cell signalling). Necrosis has previously been observed in rat liver slices following cryopreservation (Martin et al. 2000) and likely occurs following intracellular ice formation (IIF) that disrupts cell-cell contacts and plasma membranes (Karlsson & Toner 1996).

#### 4.1.1.2. Apoptosis

Apoptosis has also been shown to occur following cryopreservation (Baust, Van, & Baust 2000). In contrast to necrosis, apoptosis is programmed cell death, initiated by one or more well-defined signalling pathways. These pathways can be intracellular or extracellular. Extracellular stimuli can include cytokines and hormones or can be environmental, such as oxidative stress or supra- or sub-optimal temperature or other stresses. Oxidative stress (Fujita et al. 2005) and hypothermia (Rauen et al. 1999) have both previously been implicated as causes of injury in liver cells.

Whilst *in situ* apoptosis is unproblematic, as dying cells are phagocytosed by the host's immune system, in a BAL this process cannot occur and so apoptosis and cell fragmentation may pose a risk to the BAL recipient by releasing cell components into patients already experiencing altered inflammatory status due to the ALF. Apoptosis is now a well-understood process. This provides the potential to devise strategies to limit cell death by apoptosis. This approach has previously been proved successful for cryopreservation of primary liver cells in suspension (Matsushita et al. 2003;Yagi et al. 2001).

##### *4.1.1.2.1. Approaches to limit onset of apoptosis as a result of cryopreservation*

The process of cryopreservation has previously been shown to trigger apoptosis. Oxidative stress has been implicated as a specific cause previously but may be reduced by including antioxidants throughout the cryopreservation process. Hypothermia has also been suggested as an injury mechanism but ELS may be protected after induction by increased expression of heat shock proteins. Both of these approaches will be tested for efficacy here.

As a more general approach, methods of reducing apoptosis, regardless of cause, can be tested. Hepatocyte growth factor (HGF) is known to, among other things, protect against apoptosis but is not suitable for use here for various reasons which are described in more detail later in the Chapter. 1K1 is an engineered version of HGF which differs structurally from HGF but has similar biological activity and is readily synthesised in the laboratory. Native HGF contains an N-terminal, serine proteinase homology and 4 kringle domains (Ross et al. 2011). Conversely, 1K1 contains only 1 kringle domain as shown in Figure 4-1. 1K1 was kindly provided by Prof. Ermanno Gherardi.

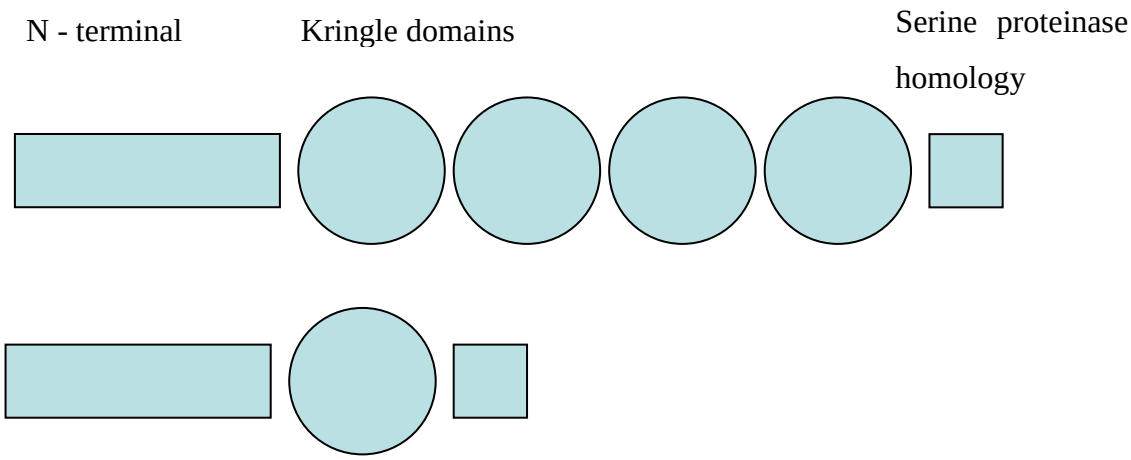


**Figure 4-1. Structures of HGF and 1K1.**

**1K1 contains only a single kringle domain, unlike HGF which contains 4.**

1K1 will be tested for efficacy at protecting ELS from apoptosis following cryopreservation.

## 4.2. Aims

The aims of this Chapter were firstly, to establish whether CIDOCD was due to either apoptosis or necrosis or both.

Following this, probable causes for the onset of CIDOCD were suggested and tested for before steps were taken to ameliorate the effects of these.

Additives were tested to show that their effects were useful and that functional recovery of cryopreserved ELS was improved by their inclusion. Where improvements were found, further investigations were made to demonstrate the mechanism of protection.

## 4.3. Methods

### 4.3.1. Cryopreservation of ELS

ELS were cryopreserved using the methods described in Chapter 2, section 2.9.2. Briefly, ELS were cooled in 12% DMSO in UW solution using a non-linear cooling profile designed to achieve a linear cooling rate of 2°C/min between -8°C and -60°C (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Antioxidants and an engineered version of hepatocyte growth factor (HGF), 1K1, were also included as additives as below.

4.3.1.1. Dose response to antioxidants

Materials

20000IU/ml catalase in 50mM phosphate buffer (pH 7.4) (40x stock)

Trolox (Vitamin E substitute that is soluble in water)

15% DMSO in UW

Method

ELS were cryopreserved using a method identical to that described in Chapter 2, section 2.9.2 except that ELS were mixed 1:4 with CPA media containing different concentrations of antioxidants, as indicated in the Results section of this Chapter.

4.3.1.2. Inclusion of antioxidants during and after cryopreservation

Materials

1.7mM Trolox and 500IU/ml catalase in 15% DMSO/UW

1.7mM Trolox and 500IU/ml catalase in complete media (ice cold and warmed)

Method

ELS were cryopreserved using a method identical to that described in Chapter 2, section 2.9.2 except that instead of mixing ELS 1:4 with CPA media, ELS were mixed 1:4 with CPA media containing antioxidants. Following cryopreservation, ELS were thawed using iced medium with or without antioxidants and returned to culture with or without antioxidants.

4.3.1.3. Inclusion of 1K1 during and after cryopreservation

Materials

100nM 1K1 in 15% DMSO/UW

100nM 1K1 in complete media

Method

ELS were cryopreserved using a method identical to that described in Chapter 2, section 2.9.2 except that instead of mixing ELS 1:4 with CPA media, ELS were mixed 1:4 with CPA media containing 1K1. Following cryopreservation, ELS were thawed and washed using iced medium with or without 1K1 and returned to culture with or without 1K1.

### 4.3.2. Hypothermic Treatment

As oxidative stress has been suggested to occur during hypothermia (without ice formation) ELS were also exposed to temperatures below 37°C for different lengths of time as indicated below.

Materials

UW solution

6-well plates

4°C fridge

Kryo 10 controlled rate freezer

Method

ELS were resuspended in UW solution at a ratio of 1:32 in 6-well plates in 8.25ml volumes. The plates were then stored in a 4°C fridge for 20 hours or at -4°C (maintained using the CRF) for 2 hours. -4°C was chosen as a low non-freezing temperature.

### 4.3.3. Hyperthermic Treatment

Hyperthermia has been suggested to increase expression of heat shock proteins (HSPSs) which may convey protection to ELS during hypothermia.

Materials

Complete culture medium

6-well plates

42°C 5% $CO_2$ humidified incubator

Method

ELS were resuspended in complete culture medium (pre-warmed to 42°C) at a ratio of 1:32 in 6-well plates in 8.25ml volumes. The plates were stored in a humidified

incubator set to 42°C for 3 hours before being returned to 37°C for 30 minutes prior to cryopreservation in an attempt to induce and allow time for expression of HSPs.

### 4.3.4. Assessing necrosis in post-thaw cultures

ELS to be assessed for necrosis were cryopreserved as in section 2.9.2. The method used to assess necrosis in post-thaw cultures was by quantification of release of lactate dehydrogenase (LDH) from membrane-damaged ELS. LDH is a stable enzyme normally present within the cytoplasm. LDH release was measured by reaction of LDH in a coupled enzymatic reaction to form a red formazan product that can be measured using a spectrophotometer at 492nm.

$$NAD^+ + \text{lactate} \rightarrow \text{pyruvate} + NADH \text{ (in the presence of LDH)}$$

$$NADH + INT \rightarrow NAD^+ + \text{formazan (red) (in the presence of diaphorase)}$$

Total LDH can be quantified by measuring LDH retained in intact ELS by subjecting ELS to multiple freeze-thaw cycles with detergent, to achieve complete cell lysis and release of LDH.

Materials

CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780)
Serum- and phenol red-free media
Lysis buffer (0.05% Triton X in PBS)
Clear-bottomed 96-well plates
Aluminium foil
-20°C freezer
Spectrophotometer at 492nm

Method

The substrate mix and assay buffer were combined and warmed to room temperature before use as per the kit instructions.

ELS were resuspended in media at 1 in 10 and mixed well. 50µl of this was placed in each well of a 96-well plate to achieve approximately 40,000 cells per well. 50µl assay buffer was added and incubated for 30 minutes in the dark at room temperature. 50µl (of red product) was then transferred to a new 96-well plate and absorbance at 492nm measured.

To release LDH retained by ELS, the remaining liquid was aspirated from ELS and 50µl of lysis buffer added to each well. The plates were placed in the -20°C freezer until frozen to -20°C, then thawed at room temperature. This process was repeated a further 2 times. 50µl assay buffer was added to each well and incubated for 30 minutes in the dark at room temperature. 50µl (of red product) was then transferred to a new 96-well plate and absorbance at 492nm measured.

The following calculations were then performed to calculate the percentage of necrotic ELS at a given time.

$$\text{Total LDH} = \text{O.D. of released LDH} + \text{O.D. of retained LDH}$$

$$\%\ \text{Necrosis} = (\text{O.D. of released LDH} / \text{O.D. of total LDH}) * 100$$

These steps were performed serially at timed intervals as indicated in the Results section of this Chapter.

### 4.3.5. Assessing of apoptosis in post-thaw cultures

ELS used to measure apoptosis in post-thaw cultures were cryopreserved in a final concentration of 12% DMSO in UW with 1.1mg/ml cholesterol. Apoptosis can be characterised by a number of morphological changes including membrane blebbing and chromatin condensation. However these morphological changes are difficult to quantify and, moreover, difficult to view within ELS.

Caspase activation is known to occur at a relatively late stage of apoptosis after initiation of apoptosis by binding of, for example, Fas ligand to receptors on the external side of the cell membrane. In particular, caspases 3, 6 and 7 are considered effector enzymes that directly cleave/activate proteins to complete the process of apoptosis.

Caspase activation can be measured using a luminescence-based assay which can be measured easily and quickly over time which is ideal for use here where rapid quantification of apoptosis is required. In the presence of caspases 3/7, a substrate containing a DEVD moiety is cleaved to produce a luminescent signal that can be measured using a luminometer (Figure 4-2).

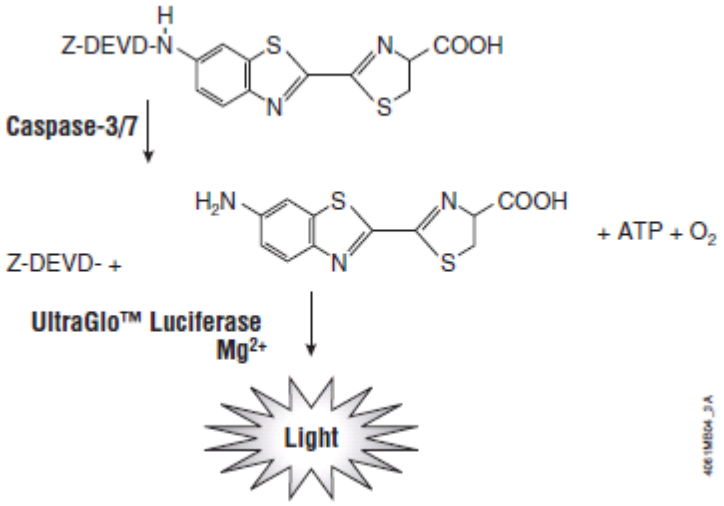


**Figure 4-2. Basis of caspase GLO assay.**

**Substrate containing DEVD is cleaved in the presence of caspase 3/7 to form a luminescent signal. Taken from "Promega Caspase Assay Technical Bulletin" 2011.**

Materials

Caspase 3/7 GLO kit (Promega, G8091)

Serum- and phenol red-free media

96-well plates (clear)

96-well white-walled plates

37°C incubator

Luminometer

Method

Caspase 3/7 GLO substrate and buffer were mixed in equal volumes and warmed to room temperature. ELS were resuspended in media at a ratio of 1 in 20, mixed well and aliquotted in 60µl volumes into each well of a 96-well plate to achieve approximately 20,000 cells per well. An equal volume of GLO reagent was added to each well and incubated for 1 hour at 37°C. 100µl was then aspirated and transferred to individual wells of a white-walled 96-well plate and luminescence measured using a luminometer. These steps were performed at timed intervals as indicated in the Results section of this Chapter.

4.3.5.1. Staurosporine treatment

Staurosporine is known to trigger apoptosis, via the intrinsic pathway, the same pathway that is thought to occur in apoptosis following cryopreservation. Staurosporine was used to measure 1K1's capability to protect against apoptosis on HepG2 cells (Feng & Kaplowitz 2002).

Materials

Complete culture medium

1µM staurosporine in serum- and phenol red-free culture medium

Serum- and phenol red-free medium

1µM staurosporine and 100nM 1K1 in serum- and phenol red-free culture medium

96-well plate

Method

HepG2 cells were resuspended in complete culture medium at a cell density of $1x10^6$/ml. Cells were aliquotted in 100µl volumes into each well of a 96-well plate to give $1x10^5$ cells per well and left to attach overnight. The next day, the medium was aspirated and cells were washed twice with serum- and phenol red-free medium. Cells were incubated with either medium alone, medium containing staurosporine or medium containing staurosporine and 1K1. Caspase activation was measured after 4 and 24 hours.

### 4.3.6. *Assessment of Oxidative Injury*

Oxidative injury was assessed using 2 methodologies, the first to quantify damaging reactive oxygen species (ROS) and the second to assess the physical damage caused to plasma membranes by these ROS.

4.3.6.1. Lipid Peroxidation Assay

Lipid peroxidation was measured at 4 hours post-warming using the Method described in Chapter 2, section 2.11.2.

4.3.6.2. Reactive Oxygen Species Assay

ROS production was measured every hour out to 4 hours post-warming using the Method described in Chapter 2, section 2.11.1.

### 4.3.7. Measuring effect of 1K1 on ELS cell growth

Method

ELS were cultured with or without 1K1 for 9 days under normal culture conditions. ELS were harvested for cell counts as described in section 2.3 on days 0, 3, 6 and 9.

### 4.3.8. Cryopreservation of ELS with optimised CPA media

Method

ELS were cryopreserved with cholesterol, antioxidants (at optimal doses) and 1K1 and returned to culture with antioxidants (at optimal doses) and 1K1. 24h post-warming, ELS were assessed for recovery and compared to an unfrozen control at equivalent time point.

### 4.3.9. Assessment of ELS Recovery in Post-thaw Cultures

4.3.9.1. Viable Cell Numbers

Viable cell numbers were assessed as described in Chapter 2 at 24 hours post-warming. As demonstrated in Chapter 3, this is when injury caused by cryopreservation is most obvious and differences between cryopreserved cohorts as assessed by all methods are most apparent.

4.3.9.2. Hepato-specific Protein Synthesis and Secretion

Hepato-specific function was assessed using ELISA as described in Chapter 2, section 2.7. As demonstrated and discussed in Chapter 3, AFP and albumin production are robust and appropriate indicators of ELS functionality and were used for this purpose in this Chapter.

## 4.4. Results

### 4.4.1. Time course of necrosis in post-warming culture

Necrosis was observed post-cryopreservation. Immediately after warming, approximately 15% of the ELS population was necrotic. At all time points thereafter, the necrotic population was increased cf. 0h ($p<0.005$) to a maximum of ~40%.

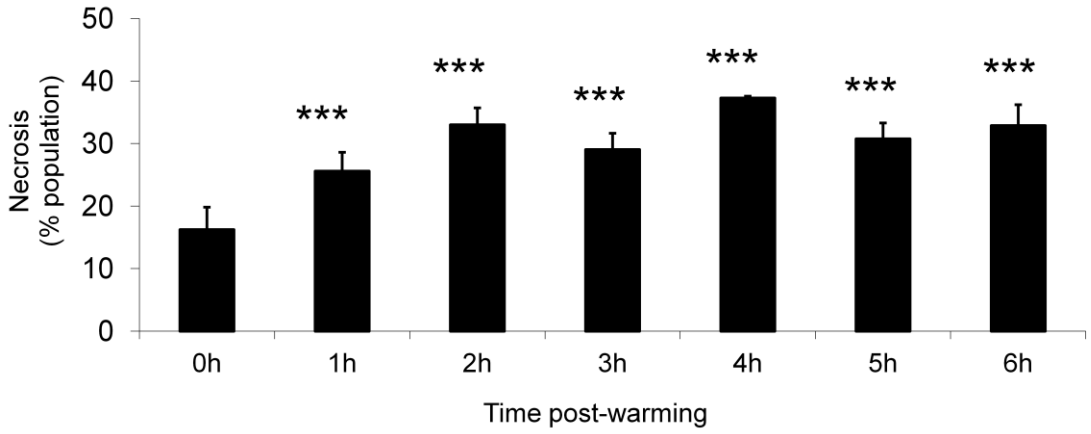


**Figure 4-3. Necrosis in post-warming cultures out to 6 hours.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW and warmed rapidly (37°C waterbath). Necrosis was measured as % LDH release between 0 and 6 hours post-warming. Data are means of n=5 +/- SD from a single experiment. ***p<0.005 compared to 0h post-warming.**

### 4.4.2. Time course of apoptosis in post-warming culture

Apoptosis was also observed following cryopreservation. Between 0 and 3 hours, caspase activation more than doubled. At 6 hours post-warming, caspase activation was greater still. By 24 hours post-warming, caspase activation had decreased compared to 3 and 6 hours post-warming but was still greater than immediately post-warming (Figure 4-4). Subsequently apoptosis was measured over a narrower time course (out to 6 hours post-warming) and was maximum at 5 hours post-warming, at approximately 5 times that immediately post-warming (Figure 4-5).

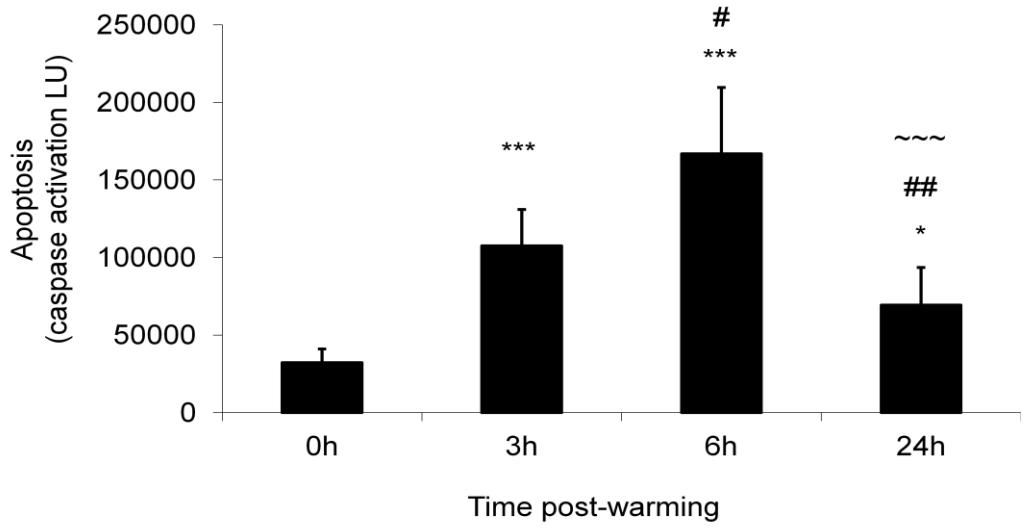


**Figure 4-4. Apoptosis in post-warming cultures out to 24 hours.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol and warmed rapidly (37°C waterbath). Apoptosis was measured at 0, 3, 6 and 24 hours post-warming. Data are means of n=6 +/- SD from a single experiment. *p<0.05, ***p<0.005 cf. 0 hours post-warming. #p<0.05, ##p<0.01 cf. 3 hours post-warming. ~~~p<0.005 cf. 6 hours post-warming.**

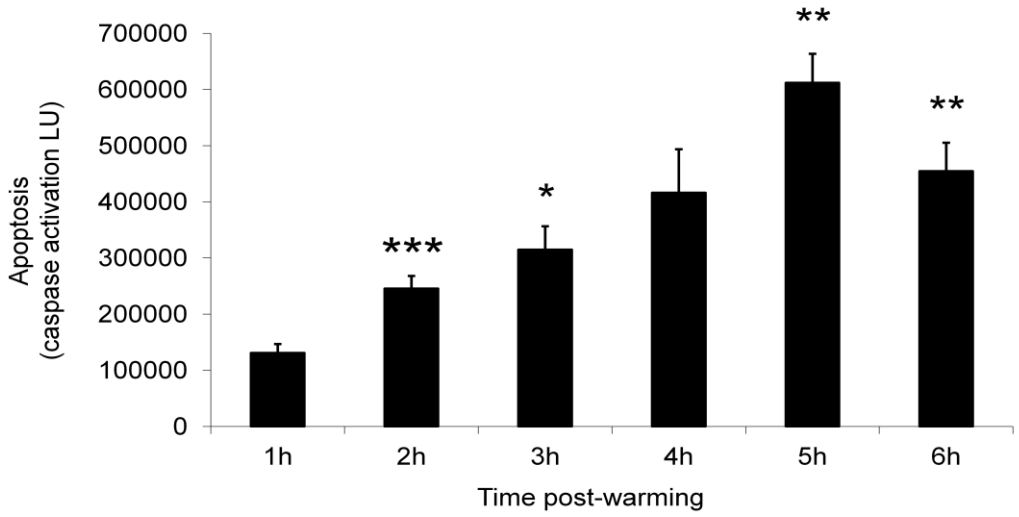


**Figure 4-5. Apoptosis in post-warming cultures out to 6 hours.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol and warmed rapidly (37°C waterbath). Apoptosis was measured every hour out to 6 hours post-warming. Data are means of n=6 +/- SD from a single experiment. *p<0.05, ***p<0.005 cf. the previous timepoint.**

### 4.4.3. Effect of Catalase during Cryopreservation

#### 4.4.3.1. Viability

ELS recovery following cryopreservation was affected by inclusion of catalase during cryopreservation in a dose-dependent fashion. Viability was improved from just below 70% (without catalase) to ~90% with 500IU/ml catalase ($p<0.05$). This experiment was repeated a further 2 times and average fold improvement in viability using 500IU/ml catalase over no catalase during cryopreservation was 1.4+/-0.23. Increasing the dose to 750IU/ml did not result in further improvement.

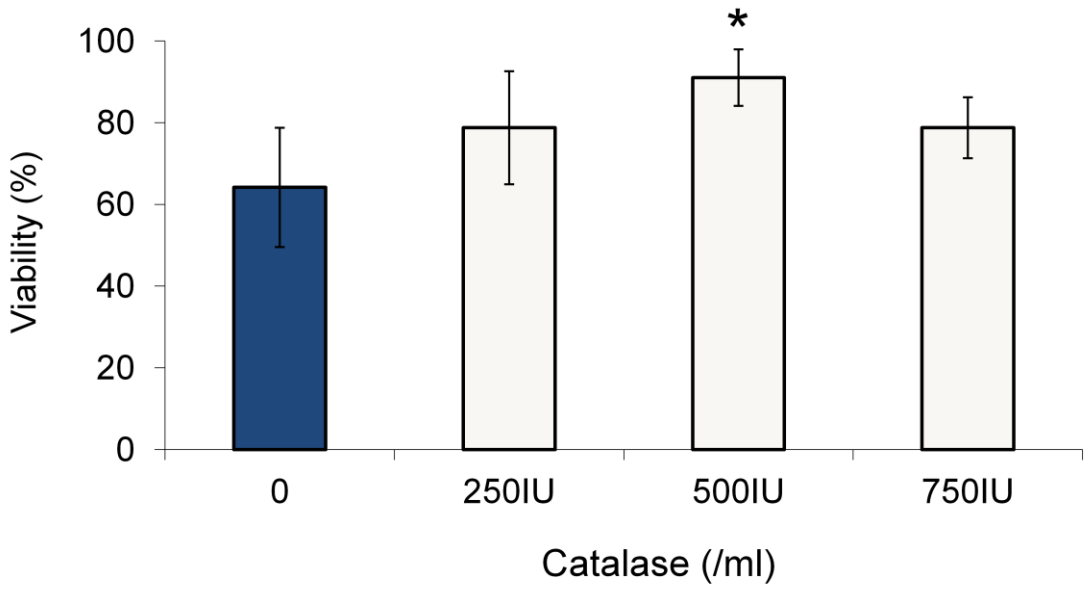


**Figure 4-6. Effect of catalase during cryopreservation on ELS viability in post-warming cultures.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with either 0, 250, 500 or 750IU catalase per ml and warmed rapidly (37°C waterbath). Data are means from n=5 +/- SD from a single experiment. *$p<0.05$ cf. ELS cryopreserved without catalase.**

#### 4.4.3.2. Viable Cell Number

Viable cell numbers were also improved in a dose-dependent manner. Again 500IU/ml catalase resulted in the greatest improvement in viable cell numbers as shown in Figure 4-7. At this concentration of catalase, viable cell numbers were increased from 1.5 million/ml to 2.1 million/ml ($p<0.05$). This experiment was repeated a further 2 times and average fold improvement was 1.3+/-0.11 (Figure 4-7).

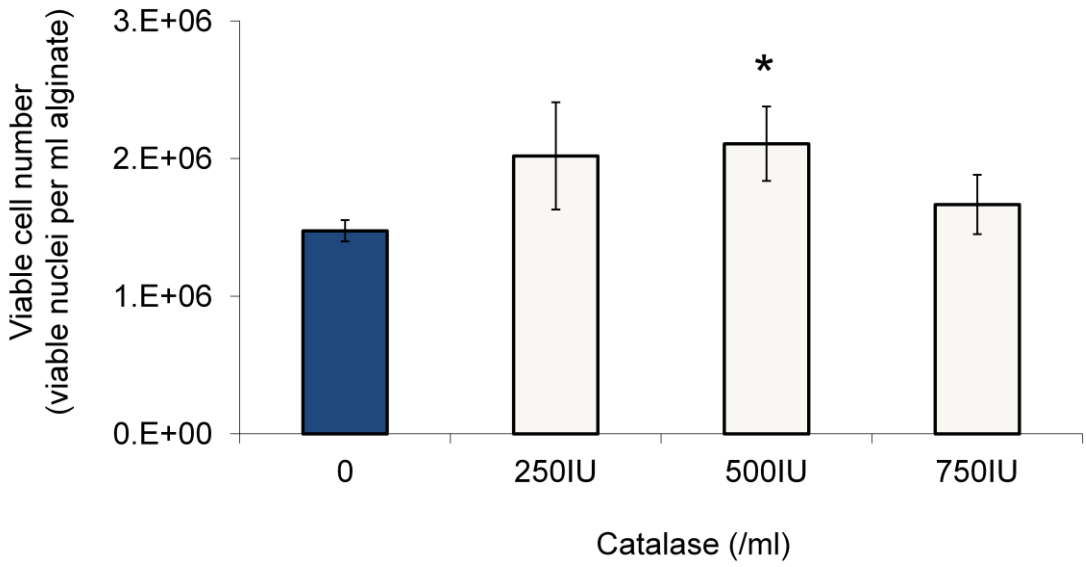


**Figure 4-7. Effect of catalase during cryopreservation on ELS viable cell numbers in post-warming cultures.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with either 0, 250, 500 or 750I U catalase per ml and warmed rapidly (37°C waterbath). Data are means of n=5 +/- SD from a single experiment. *p<0.05 cf. ELS cryopreserved without catalase.**

### 4.4.4. Effect of Trolox during Cryopreservation

#### 4.4.4.1. Viability

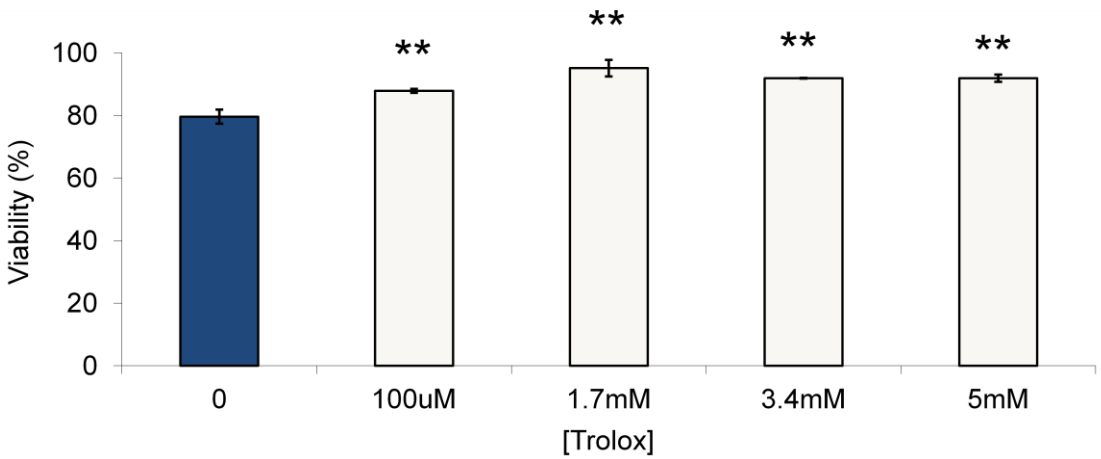


**Figure 4-8. Effect of Trolox during cryopreservation on ELS viability in post-warming cultures.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with either 0, 100µM, 1.7mM, 3.4mM or 5mM Trolox and warmed rapidly (37°C waterbath). Data are means of n=5 +/- SD from a single experiment. **p<0.01 cf. ELS cryopreserved without Trolox.**

Cryopreservation of ELS with different concentrations of Trolox resulted in improved ELS viability at all concentrations cf. without Trolox ($p<0.01$). However, viability was maximally improved using 1.7mM Trolox. At this concentration, ELS viability was improved from 79% without Trolox to 95% with 1.7mM Trolox ($p<0.01$) (Figure 4-8 above). This experiment was repeated a further 2 times and by using 1.7mM Trolox during cryopreservation and an average fold improvement in ELS viability of 1.3+/-0.3 was found.

4.4.4.2. Viable cell number

Viable cell numbers were affected by inclusion of Trolox in a dose-dependent fashion. At the highest dose of Trolox (5mM), viable cell numbers were reduced compared to ELS cryopreserved without Trolox. However, using the dose that gave the most improved viability (1.7mM), viable cell numbers were significantly improved cf. ELS cryopreserved without Trolox ($p<0.05$). This experiment was repeated a further 2 times and average fold improvement was 1.2+/-0.3 when 1.7mM Trolox was used.

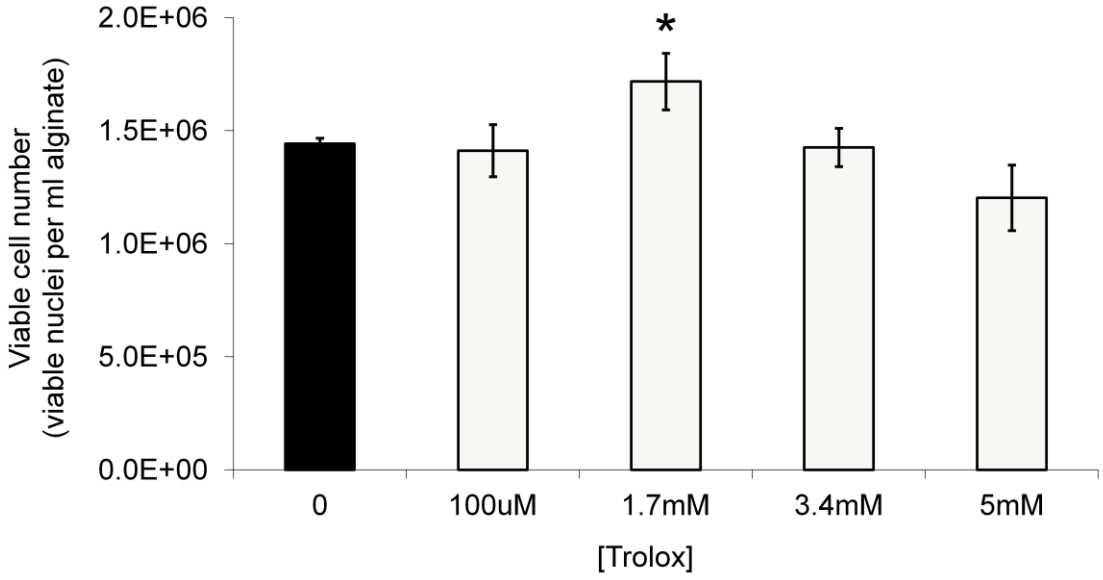


**Figure 4-9. Effect of Trolox during cryopreservation on ELS viable cell numbers in post-warming cultures.**

**ELS were cooled using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with either 0, 100µM, 1.7mM, 3.4mM or 5mM Trolox and warmed rapidly (37°C waterbath). Data are means of n=5 +/- SD from a single experiment. *p<0.05 cf. ELS cryopreserved without Trolox.**

### 4.4.5. Does hypothermia cause oxidative stress?

4.4.5.1. Storage at 4°C for 20 hours

*4.4.5.1.1. Reactive Oxygen Species Production*

For all cohorts, ROS production increased over time. ELS stored at 4°C for 20 hours without antioxidants showed increased ROS production post-thawing cf. ELS maintained at 37°C. This was true at all time points measured but was only significant immediately after hypothermia ($p<0.05$). At all time points ELS stored at 37°C with antioxidants showed decreased ROS production when compared to those stored at 37°C without antioxidants although this was not statistically significant. ELS stored at 4°C without antioxidants produced 0.89 and 1.15nmoles hydrogen peroxide per mg protein after 3 and 4 hours respectively. However, by including antioxidants, hydrogen peroxide production of ELS stored at 4°C was significantly reduced (0.74 and 0.99nmoles per mg protein at 3 and 4 hours respectively) ($p<0.05$).

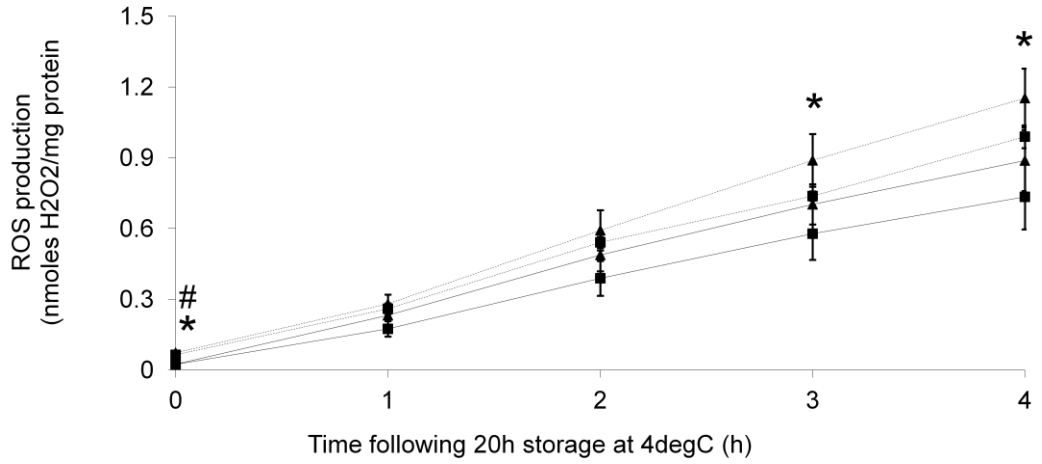


**Figure 4-10. ROS production following ELS storage at 4°C for 20 hours.**

**ELS were stored at either 4°C (dashed lines) or 37°C (solid lines) either with (■) or without (▲) antioxidants for 20 hours before ROS production was measured out to 4 hours post-treatment. Results are normalised to mg protein. Data are means of n=4 +/- SD from a single experiment. #p<0.05 between ELS stored at 4°C and 37°C (both without antioxidants). *p<0.05 between ELS stored at 4°C with or without antioxidants.**

*4.4.5.1.2. Oxidative Damage*

Oxidative damage, assessed by measurement of malondialdehyde production, was greatest for ELS stored at 4°C without antioxidants (4 media) at ~0.4µmoles per mg protein. This was significantly higher than ELS stored at 37°C with and without

antioxidants and also than ELS stored at 4°C with antioxidants ($p<0.05$). There was no significant difference between the remaining 3 cohorts.

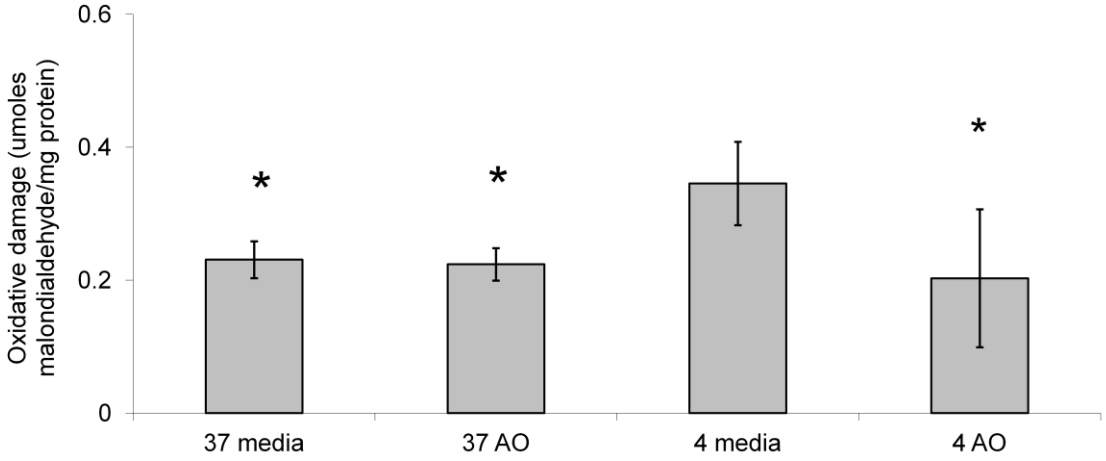


**Figure 4-11. Oxidative damage following ELS storage at 4°C for 20 hours.**

**ELS were stored at either 37°C or 4°C with (AO) or without (media) antioxidants. Results are normalised to mg protein. Data are means of n=5 +/- SD from a single experiment. *p<0.05 compared to ELS stored at 4°C without antioxidants.**

4.4.5.2. Storage at -4°C for 2 hours

*4.4.5.2.1. Reactive Oxygen Species (ROS) Production*

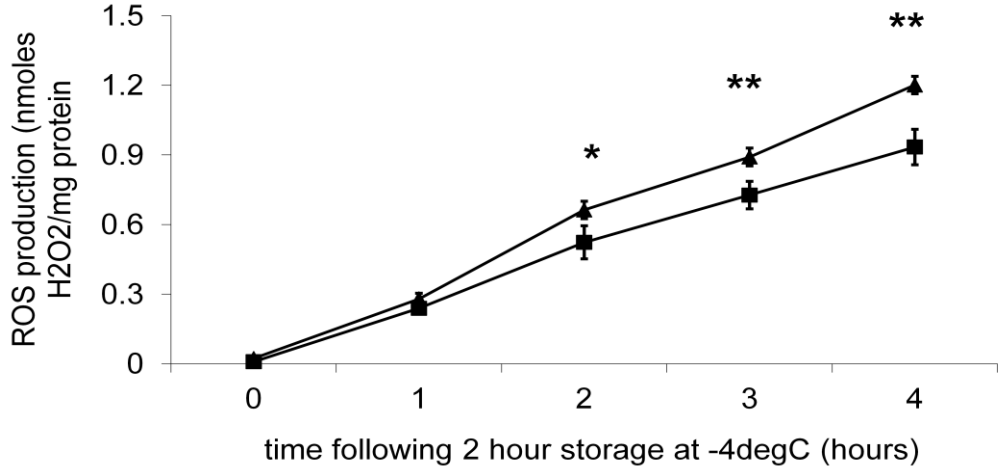


**Figure 4-12. ROS production following ELS storage at -4°C for 2 hours with or without antioxidants.**

**ELS were stored either with (■) or without (▲) antioxidants for 2 hours before ROS production was measured out to 4 hours post-treatment. Results are normalised to mg protein. Data are means of n=4 +/- SD from a single experiment. *p<0.05, **p<0.01 cf. ELS stored with antioxidants.**

ROS production again increased with time. ELS stored at -4°C without antioxidants showed increased ROS production cf. those stored with antioxidants at all time points. However, this change was only significant at the later time points measured (2, 3 and 4 hours post-treatment). On average, over the entire time course, ROS production was ~1.2+/-0.04-fold greater in ELS stored without antioxidants cf. those stored with antioxidants (Figure 4-12).

*4.4.5.2.2. Oxidative damage*

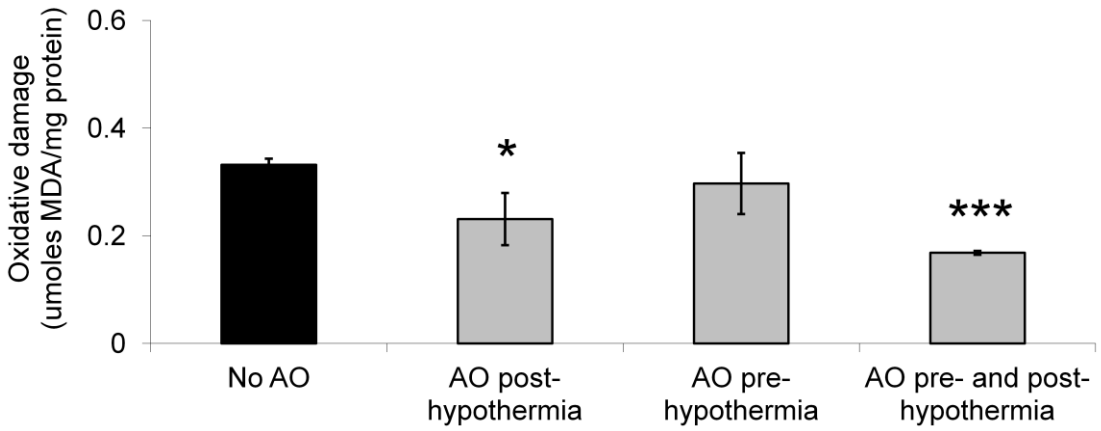


**Figure 4-13. Oxidative damage following ELS storage at -4°C for 2 hours.**

**Antioxidants (AO) were administered either pre-hypothermia or not. Following hypothermia, ELS were returned to culture either with or without AO. Results are normalised to mg protein. Data are means of n=5 +/- SD from a single experiment. *p<0.05, ***p<0.005 compared to ELS stored at -4°C without AO.**

Oxidative damage was greatest in ELS stored at -4°C without antioxidants. None of the samples had nucleated; this was confirmed visually. A significant reduction in oxidative damage was achieved by including antioxidants after hypothermia (but not during). This reduction was from 0.33 to 0.23 µmoles MDA/mg protein, n=5 ($p<0.05$). Oxidative damage was unaffected when antioxidants were included during hypothermia but not during warming. However, the greatest reduction was achieved when antioxidants were used both during and after hypothermia. Using this protocol, oxidative damage was reduced to 0.17 µmoles MDA/mg protein, n=5, $p<0.005$.

### 4.4.6. Does cryopreservation cause oxidative stress?

#### 4.4.6.1. Reactive Oxygen Species Production

ELS cryopreserved with or without antioxidants both displayed increasing ROS production with time out to 4 hours post-warming. ELS cryopreserved with antioxidants showed reduced ROS production per mg protein when compared to those cryopreserved without antioxidants. This was true at all time points but only statistically significant at all time points after 0h (immediately post-warming) ($p<0.005$). On average over all time points, ROS production was reduced by approximately 3-fold.

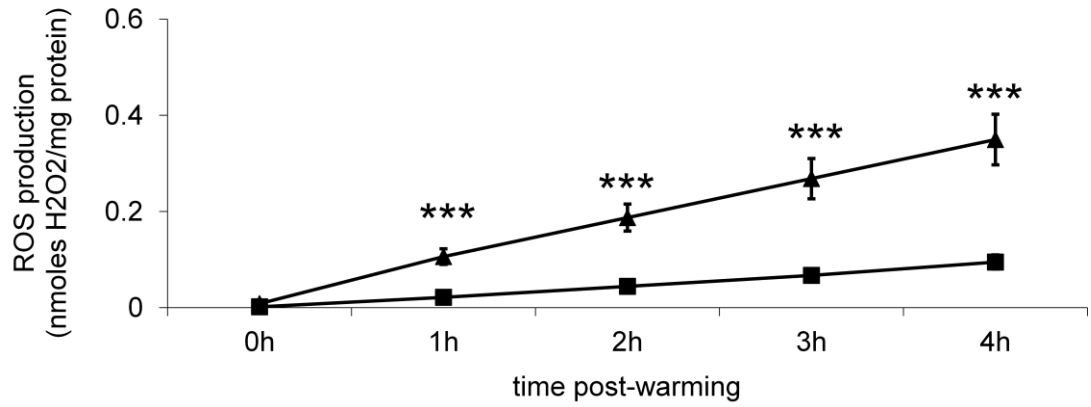


**Figure 4-14. ROS production following ELS cryopreservation.**

**ELS were cryopreserved using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW either with (■) or without antioxidants (▲) and warmed rapidly (37°C waterbath). Results are normalised to mg protein. Data are means of n=4 +/- SD from a single experiment. ***p<0.005 cf. ELS cryopreserved with antioxidants.**

#### 4.4.6.2. Oxidative Damage

Oxidative damage was greatest for ELS cryopreserved without antioxidants at approximately 0.5µmoles MDA per mg protein. By administering antioxidants before **or** after cryopreservation, oxidative damage was reduced, although this was not statistically significant. However, when antioxidants were present during **and** after cryopreservation, oxidative damage was halved to 0.24µmoles MDA per protein ($p<0.05$) (Figure 4-15 below).

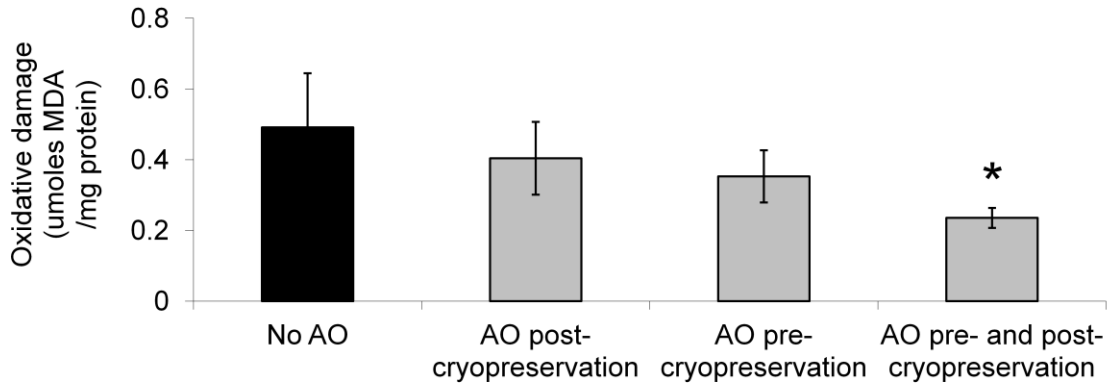


**Figure 4-15. Oxidative damage following ELS cryopreservation.**

**ELS were cryopreserved using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW and warmed rapidly (37°C waterbath). AO were administered either pre- or post- or both pre- and post- cryopreservation. Results are normalised to mg protein. Data are means of n=5 +/- SD from a single experiment. *p<0.05 cf. ELS cryopreserved without antioxidants.**

### 4.4.7. Hyperthermic treatment

Hyperthermic treatment (42°C for 3 hours prior to cryopreservation) did not affect ELS recovery. Although both viable cell number and total function were very slightly improved by hyperthermic treatment this was not statistically significant (NS). The results at 24 hours post-warming are given in Table 4-1 below. Data are means of n=5 +/- SD from a single experiment.

**Table 4-1. Effect of hyperthermic treatment on ELS recovery in post-warming cultures.**

| | No treatment | Hyperthermia | Statistics |
|---|---|---|---|
| Viable cell number ($10^6$ per ml alginate) | 3.8 +/- 0.2 | 4.0 +/- 0.7 | NS |
| Albumin production (µg/well/24h) | 33 +/- 5 | 34 +/- 5 | NS |

### 4.4.8. Can 1K1 protect against staurosporine-triggered apoptosis?

ELS were treated for either 4 or 24 hours with staurosporine to trigger initiation of apoptosis. After 4 hours, although caspase activation was increased in cells treated with staurosporine compared to the media control, it did not reach statistical significance. However, when HepG2 cells were treated overnight with staurosporine a significant 6-

fold increase in caspase activation was seen ($p<0.005$). This was entirely ameliorated when 1K1 was included (along with staurosporine); caspase activation did not occur.

In agreement with these findings, morphology of HepG2 cells plated in medium remained normal over the treatment time. Cells stayed flattened, with no evidence of detachment. However, HepG2 cells treated with staurosporine showed an altered morphology: cells were rounded up and in some cases had detached. Additionally, some cells contained increased numbers of vacuoles. When 1K1 was included along with staurosporine this altered morphological state was not observed and cells retained a good morphology comparable to that of the media control (Figure 4-16 below).

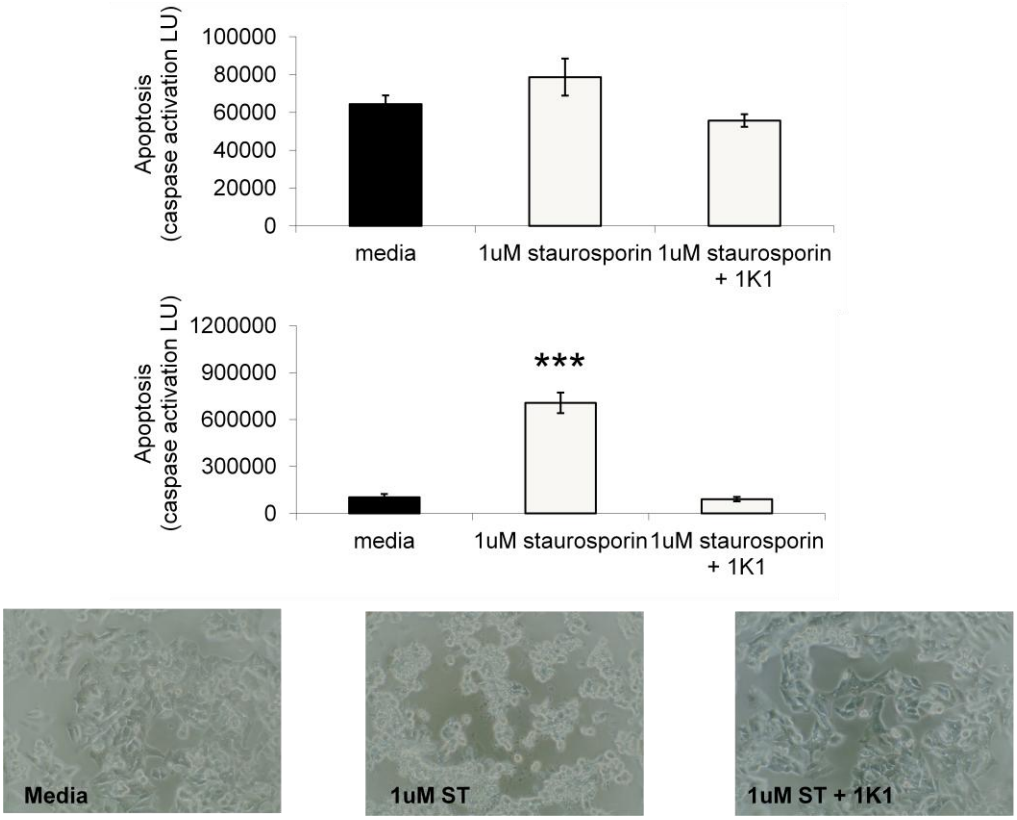


**Figure 4-16. Protective effect of 1K1 against staurosporine.**

**HepG2 cells were treated with staurosporine alone or with 1K1 for either 4 hours (top) or overnight (middle) and caspase activation measured. Data are means of n=8 +/- SD from single experiments. ***p<0.005 cf. media control. Phase images show morphology of HepG2 cells in normal media compared with HepG2 cells treated with staurosporin alone (ST – bottom middle) or with 1K1 (bottom right). Original magnification x20.**

### 4.4.9. Can 1K1 protect against apoptosis caused by cryopreservation?

4.4.9.1. Effect of 1K1 when included during cryopreservation

For both cryopreserved cohorts, caspase activation increased out to 3 hours post-warming and levelled off thereafter. When 1K1 was included during cryopreservation, and caspase activation was measured out to 6 hours in post-warming cultures, it was significantly lower at all time points measured. On average, over all the time points, caspase activation was 1.5-fold greater when 1K1 was not included during cryopreservation (Figure 4-17).

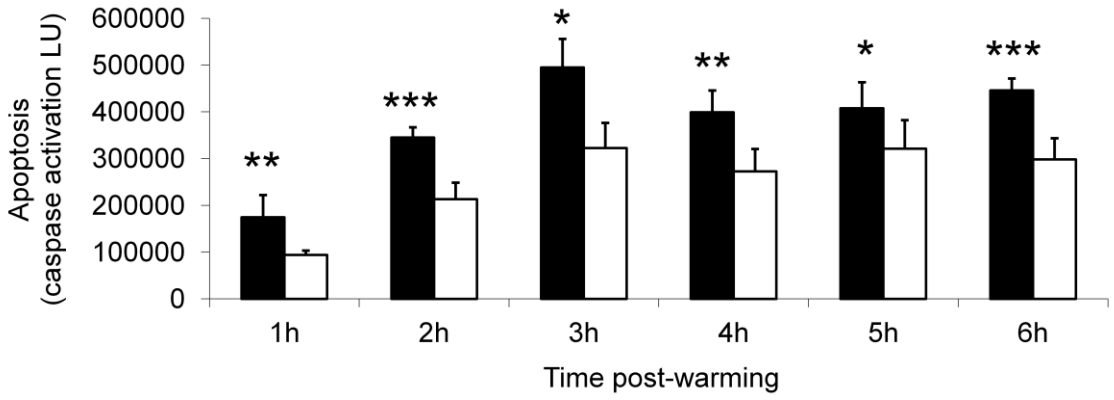


**Figure 4-17. Effect on apoptosis in post-warming cultures of inclusion of 1K1 during cryopreservation.**

**ELS were cryopreserved using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol and warmed rapidly (37°C waterbath). Caspase activation out to 6h post-warming was measured in ELS cryopreserved with (white bars) or without (black bars) 1K1. Data are means of n=6 +/-SD from a single experiment. *p<0.05, **p<0.01, ***p<0.005 compared to ELS cryopreserved without 1K1 at the same time point.**

4.4.9.2. Effect of 1K1 when included during cryopreservation and in post-warming cultures

Again, for both cryopreserved cohorts, caspase activation increased out to 3 hours post-warming before levelling off. When 1K1 was included during cryopreservation and in post-warming cultures, caspase activation was reduced at all time points measured. On average, caspase activation from ELS cryopreserved/cultured without 1K1 was 1.7-fold greater than ELS cryopreserved/cultured with 1K1.

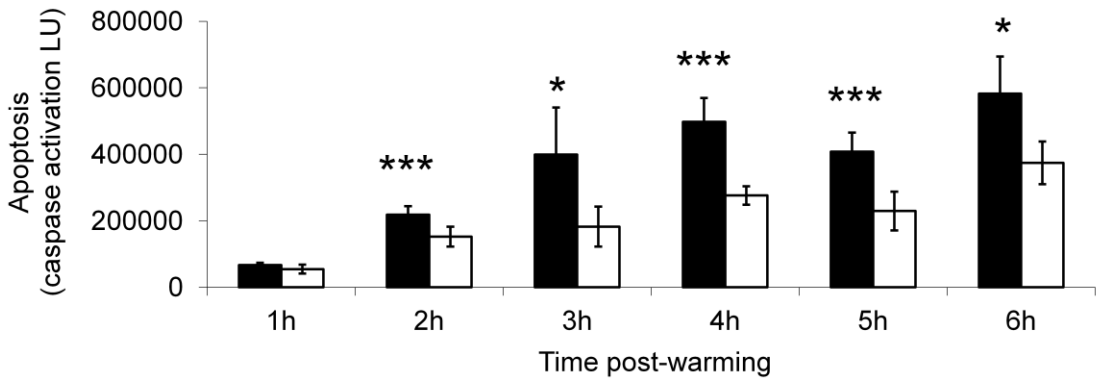


**Figure 4-18. Effect on apoptosis in post-warming cultures of inclusion of 1K1 during cryopreservation and post-warming cultures.**

**ELS were cryopreserved using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol and warmed rapidly (37°C waterbath). Caspase activation out to 6h post-warming was measured in ELS cryopreserved/cultured with (white bars) or without (black bars) 1K1. Data are means of n=6 +/-SD from a single experiment. *p<0.05, ***p<0.005 compared to ELS cryopreserved/cultured without 1K1 at the same time point.**

1K1 also reduced necrosis in post-thaw cultures at 0, 3 and 6 hours post-warming although this was only significant at 3h.

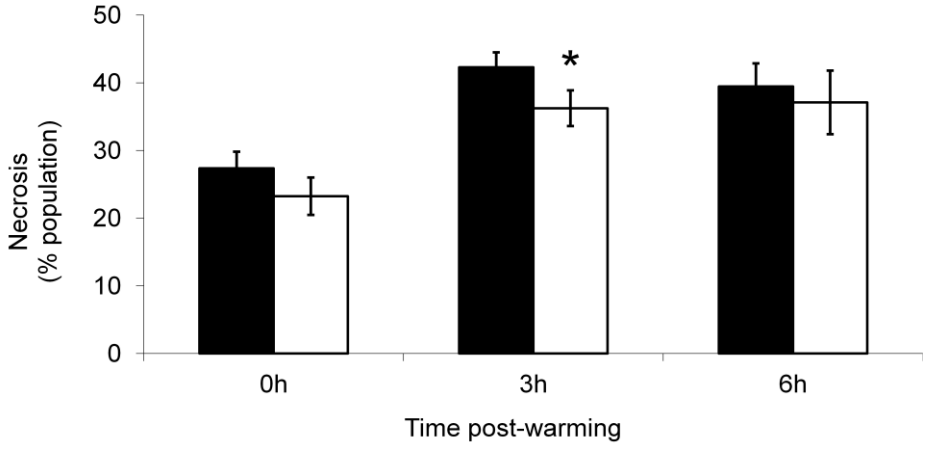


**Figure 4-19. Effect of inclusion of 1K1 during cryopreservation and post-warming cultures on necrosis.**

**ELS were cryopreserved using a non-linear cooling profile (Diener et al. 1993) in 12% DMSO/UW and warmed rapidly (37°C waterbath). Necrosis out to 6h post-warming was measured in ELS cryopreserved/cultured with (white bars) or without (black bars) 1K1. Data are means of n=5 +/-SD from a single experiment. *p<0.05 compared to ELS cryopreserved/cultured without 1K1 at the same time point.**

### 4.4.10. Does 1K1 improve ELS recovery following cryopreservation?

Viable cell numbers were significantly improved when 1K1 was included during cryopreservation whilst per cell synthetic function was slightly improved (although not significantly (NS)). These results are summarised in Table 4-2 below. Data are means of n=5 +/- SD from a single experiment.

**Table 4-2. Effect of 1K1 on ELS recovery in post-warming cultures.**

| | (CPA) media | 1K1 | Statistics |
|---|---|---|---|
| Viable cell number ($10^6$ per ml alginate) | 4.2 +/- 0.17 | 4.7 +/- 0.2 | $p<0.05$ |
| Albumin production (µg/million nuclei/24h) | 5.86 +/- 0.29 | 6.28 +/- 0.19 | NS |

### 4.4.11. Does 1K1 affect cell growth rates?

When ELS were cultured with or without 1K1 from immediately after encapsulation out to 9 days post-encapsulation, there was no difference in growth at any time point.

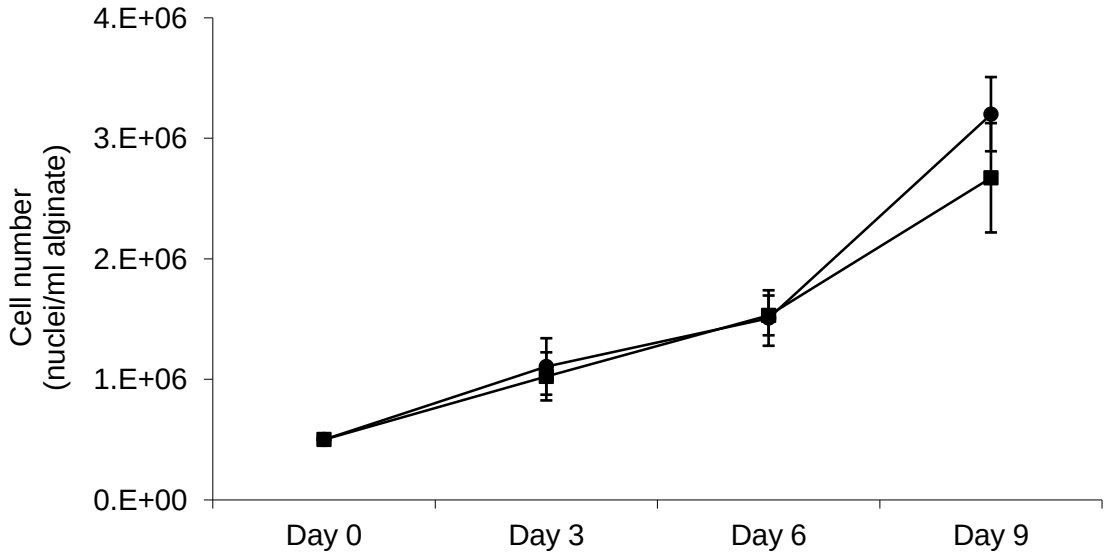


**Figure 4-20. Effect of 1K1 on ELS proliferation.**

**ELS were cultured with (■) or without 1K1 (●) and proliferation measured on days 3, 6 and 9 post-encapsulation. Data are means of n=5 +/- SD from a single experiment.**

### 4.4.12. Recovery of ELS cryopreserved using optimal CPA media

Viability of the cryopreserved ELS was approximately 5% lower than that of the unfrozen control ($p<0.05$). Viable cell numbers of the cryopreserved cohort was also reduced compared to the unfrozen control by 20% ($p<0.05$). Function in cryopreserved ELS was reduced further by nearly 30% compared to the unfrozen control (not statistically significant). These results are summarised in Table 4-3 below.

**Table 4-3. Recovery of ELS cryopreserved using optimal CPA media at 24hours post warming.**

| | Unfrozen ELS | Optimally cryopreserved ELS | Statistics |
|---|---|---|---|
| Viability (%) | 99.2 +/- 1.4 | 94.0 +/- 2.6 | $p<0.05$ |
| Viable cell number ($10^6$ per ml alginate) | 9.9 +/- 0.8 | 8.0 +/- 0.5 | $p<0.05$ |
| Albumin production (µg/well/24h) | 85.8 +/- 18.6 | 62.2 +/- 9.4 | $p=0.72$ |

Although a direct comparison between the starting and optimal cryopreservation protocols was not made within the same experiment, results from Chapter 3 show that using the starting protocol viability was reduced by ~60%, viable cell numbers by 70% and function by 80% at 24 hours post-warming. These results demonstrate that the optimised protocol results in greatly improved ELS recoveries in post-thaw cultures.

## 4.5. Discussion

In the previous Chapter, it was demonstrated that intracellular ice formation (IIF) and propagation was lethally damaging to ELS. Including cholesterol as a heterogeneous nucleator to limit supercooling and reduce incidence of intracellular ice formation was effective in reducing injury via this mechanism. However, despite these improvements, recovery was still likely insufficient for ELS to be used within a BAL. Therefore, the aim of this Chapter was to identify other, more subtle, potential mechanisms of injury that damage ELS during cryopreservation, and attempt to prevent or treat them.

Both necrosis and apoptosis are apparent following cryopreservation. Necrosis is commonly observed following cryopreservation in various cell/tissue types including heart valves (Vazquez et al. 2008) and liver (Martin et al. 2000). No published literature could be found regarding the timescale of necrosis following cryopreservation in liver cells although in a similar cell type (kidney cell line), necrosis was greatest at 6 hours post-warming (in that study necrosis was measured at 1, 6, 12, 24 and 48 hours post-warming) (Baust et al. 2001). For ELS, maximum necrosis occurred at 3 hours and levelled off thereafter and so ELS data seems comparable to that study.

In my study, necrosis was quantified using lactate dehydrogenase (LDH) release. Uncontrolled release of LDH will occur once the plasma membrane has been breached or permeabilised. In ELS, we know that cryopreservation results in a loss of membrane integrity (probably as a result of IIF) demonstrated by positive staining for propidium iodide (PI) as presented in Chapter 3. This damage will occur during freezing and thawing and so this timescale (3-6 hours post-thawing) of injury seems appropriate.

Similarly, an apoptotic response following cryopreservation has also been observed in other cell types, including sperm (Khan et al. 2009; Martin et al. 2007) and embryonic stem cells (Xu et al. 2010) but also liver (Fujita et al. 2005; Vanhulle et al. 2006) both as single cell suspensions and as tissue sections. For ELS, maximum caspase activity following cryopreservation was between 3 and 6 hours, and diminished by 24 hours post-warming. This timescale is in agreement with findings from others: in one study using cryopreserved porcine hepatocytes, caspase activity was measured immediately post-warming and then at 6 and 24 hours post-warming and was greatest at 6 hours post-warming (Matsushita et al. 2003); in another study using rat hepatocytes, caspase activity was apparent at 3 hours post-warming (Mahler et al. 2003); furthermore, Baust et al. used MDCK cells and found that by 24 hours post-warming, caspase activation

had returned to the low levels quantified immediately after cryopreservation (Baust et al. 2001): also in agreement with ELS findings here. This may correlate fragmentation of the injured cells at later time points with loss of the enzyme.

Apoptosis is triggered by one of two pathways: intrinsic or extrinsic. The extrinsic pathway requires binding of a death receptor on the external cell membrane by, for example, Fas ligand. This causes cleavage/activation of caspase 8 which then activates caspase 3. In contrast, the intrinsic pathway results from various environmental stimuli such as presence of reactive oxygen species (ROS), hypoxia (Vanhulle et al. 2006) and hypothermia/reperfusion (Duval et al. 2006). These stimuli are all well-aligned with events that occur during the cryopreservation process and so the apoptotic response of ELS is not surprising. ROS have been proposed as apoptosis-triggering in both *in vivo* liver injury studies (San Miguel et al. 2006) and also in *in vitro* studies using HepG2 cells (Oh & Lim 2006). Any of these stimuli can result in mitochondrial alterations which lead to cytochrome c release, followed by caspase 9 activation which activates caspase 3. Caspase 3 is common to both the intrinsic and extrinsic pathway (Slee et al. 1999) and moreover, is considered to be an effector caspase (Bilodeau 2003).

Effector protein (including caspase 3) activation occurs late in the well-defined apoptotic cascade and immediately precedes events that are characteristic of apoptosis. This has been demonstrated in 3 liver cell lines including HepG2 cells (Chen et al. 2002). Apoptosis can be observed using microscopy and can manifest as decreased cell volume and/or condensation or fragmentation of nuclei prior to whole cell fragmentation (Bilodeau 2003). However, these effects are not always observed and could be considered subjective, making objective quantification difficult. Moreover, in ELS, where cells are in a 3D culture format, these changes may be challenging to view. Caspase 3 activation does not confirm completion of apoptosis. Downstream of caspase 3 activation, cleavage of poly (ADP-ribose) polymerase (PARP) occurs and this is considered to confirm apoptosis. This could be quantified using Western blot or ELISA and would provide certainty that apoptosis had occurred in ELS here. Despite this, caspase 3 activation remains a reliable indicator of progression of apoptosis and can be easily quantified over time in this study which is advantageous.

Since ROS production was indicated as a potential injurious mechanism resulting in apoptosis and cell death, antioxidants were trialled to see if ELS recovery could be improved. Both catalase and Trolox (water-soluble vitamin E) were found to be

beneficial and both significantly improved ELS recovery, implicating that ELS do suffer the effects of oxidative stress as a result of cryopreservation.

Catalase is a cytoplasmic protein and catalyses conversion of hydrogen peroxide to water and oxygen. Hydrogen peroxide is produced after dismutation of superoxide by superoxide dismutase. No literature could be found regarding the use of catalase for liver cell cryopreservation. However, many studies describe the beneficial impact of catalase for cryopreservation of sperm cells from various species (Michael et al. 2007; Paudel et al. 2010; Roca et al. 2005). Concentrations of catalase used in these studies were 200, 1200 and 400IU/ml respectively. ELS recovery was optimally improved using 500IU/ml catalase which is comparable to the other studies. Due to the methods of assessing recovery in these other studies, it is difficult to assess how "useful" catalase is for ELS compared to sperm cells. Liver cells, including HepG2 cells, do produce catalase and so it would seem reasonable to suggest that this protective mechanism is still active in ELS (and indeed may be increased in ELS along with other functions) but may be supplemented to protect further.

Trolox, vitamin E, is lipophilic and closely associated with plasma membranes. Trolox is preferentially oxidised over poly-unsaturated fats in the plasma membrane and this provides its protective mechanism. Again, no literature could be found regarding the use of Trolox in liver cell cryopreservation protocols. However, Trolox has been shown to improve recovery of sperm cells as assessed using parameters including motility and mitochondrial membrane potential (Pena et al. 2004; Thuwanut et al. 2008). Unlike catalase, Trolox appears to be cytotoxic (as indicated by reduced viable cell numbers compared to the control when 5mM Trolox was used). It may be that due to Trolox's association with the plasma membrane, prolonged exposure may lead to irreversible damaging alterations to ELS. These observations also raise an interesting point for cryopreservation strategies that interventions with antioxidants can have both positive and negative effects depending on dose and exposure time. Thus "blanket" application of antioxidants, without suitable dose range studies or consideration of the stage of cryopreservation at which they are administered, may not result in improvements (Dai & Meng 2011).

Hypothermia (at 4°C overnight) resulted in increased ROS production and lipid peroxidation compared to overnight storage at 37°C but was ameliorated using antioxidants. Hypothermia is known to result in oxidative stress. When rat hepatocytes

were stored at 4°C before being returned to culture for an hour, lipid hydroperoxides and TBARs were increased by up to 10-fold compared to an untreated control (Rauen et al. 1999). In a similar study using rat hepatocytes, reduced intracellular glutathione content was proposed as the reason for increased oxidative stress but, again, could be corrected using antixoxidants (N-acetyl-cysteine) (Vairetti et al. 2001). An alternative suggested pathway in kidney cells stored at 4°C overnight is activation of Rac1 which activates NADPH to produce superoxide, resulting in oxidative stress (and also initiates MEK/ERK pathway) (Karhumaki et al. 2007). Finally, an alternative proposed mechanism is that of cold-induced iron release. Huang et al. found that iron release from kidney cells increased with storage time at 4°C (out to 72 hours) (Huang & Salahudeen 2002). This iron is then free to partake in Fenton chemistry, resulting in continued ROS production.

No literature could be found regarding sub-zero storage of cells and the impact (or not) on ROS production and oxidative damage thereafter. However, it would seem reasonable that oxidative stress would increase with time and decreasing temperature. Here, ELS were stored at -4°C for 2 hours as this is the length of time that may be required to cool ELS in large volumes as would eventually be required to treat an adult patient with ALF. Under these conditions, oxidative stress could still be ameliorated using antioxidants with levels of MDA returning to those comparable to ELS stored at 37°C (~0.2µmoles/mg protein). As oxidative stress can be counteracted and improvements in ELS recovery observed, this would suggest that this antioxidant protocol was sufficient to protect against oxidative damage.

Cold shock (temperature change) is often proposed as an injurious mechanism that results in poor recovery of cells following cryopreservation. Heat shock protein (HSP) expression has been shown to be reduced in renal tubular cells with prolonged (beyond 16 hours) hypothermia (although HSP90 expression was reduced sooner – after 8 hours hypothermia) (Healy et al. 2006). Heat shock proteins are chaperone proteins that assist in protein-protein interactions and stabilise partially unfolded proteins. We know that ELS suffer from oxidative stress which is known to damage proteins and so the decreased expression of HSP during hypothermia may be damaging to ELS as these HSP functions are not being performed.

Stimulating upregulation of these HSPs prior to cryopreservation may improve ELS recovery. Many groups have investigated HSP with relation to cold storage (without

cryopreservation). HSP72 expression may be increased using chemical induction using doxorubicin in rat liver and this was shown to result in improved cellular morphology, and reduced levels of liver injury markers compared to uninduced cells after up to 72 hours hypothermia (Chen et al. 2004). Alternatively, HSP72 expression can be induced by hyper- (and to a lesser degree hypo-) thermia in HepG2s (Rada et al. 2005) and renal tubular cells (Healy et al. 2006). Furthermore, upregulation of HSPs has been shown to reduce apoptosis in renal cells (Healy et al. 2006) and improve recovery (LDH activity) of a liver cell line (Kao et al. 2008).

Here, however, ELS recovery following cryopreservation was not improved significantly by hyperthermia. There are a number of potential explanations for this. Firstly, and most likely, is that HSPs are not down-regulated with cryopreservation as the process is too quick. The studies described above showed decreased HSP expression following only prolonged hypothermia. Alternatively, the HSP hyperthermia induction method here may be unsuitable/sub-optimal for ELS. Another point for consideration is that ELS already show increased stress protein expression (as a result of 3D culture format) and further induction is impossible (Choudhury et al. 2003). These possibilities could be assessed using Western blot analysis to either confirm or disprove them.

As a more general approach, any stress that ELS are exposed to during the cryopreservation process (e.g. oxidative, osmotic, hypoxia, cold etc) may result in apoptosis and this has been described previously. Therefore, another approach trialled here was the use of 1K1, an engineered version of hepatocyte growth factor (HGF). *In vivo* HGF is known to be morphogenic, mitogenic and capable of promoting cell survival (Huh et al. 2004; Stoker et al. 1987; Zhang & Woude 2003). *In vitro* HGF has been shown to be an effective anti-apoptotic agent for adult human hepatocytes (Saich et al. 2007;Schulze-Bergkamen et al. 2004). However, HGF is expensive to produce biologically as it requires expression in mammalian cells (Park et al. 2010). 1K1 is an engineered version of HGF that differs structurally and can be produced from yeast cells, making it more cost-effective than 1K1. Importantly, 1K1 retains signalling activity both *in vivo* and *in vitro* (Ross et al. 2009; Roy et al. 2010).

Before 1K1 was tested within the ELS cryopreservation protocol, its efficacy was tested against apoptosis caused by staurosporine. The exact mechanism by which staurosporine causes apoptosis remains elusive. Classically, staurosporine was thought

to act by triggering cytochrome c release from mitochondria that then forms a complex with Apaf-1 that activates caspase 9, which activates effector caspase 3 (Feng & Kaplowitz 2002). More recently though, staurosporine has been shown to act via an alternative apoptosis pathway, still via caspase 9 but independently of Apaf-1 (Manns et al. 2011). Either way, it is agreed that staurosporine represents a good model for intrinsic apoptosis (the same pathway which would be initiated during cryopreservation) and is, therefore, an appropriate pro-apoptotic for 1K1 to be tested against.

Staurosporine resulted in increased levels of caspase activation with increasing exposure time and was associated with poor morphology, characteristic of apoptotic cells after overnight exposure. However, when 1K1 was given at the same time as staurosporine, these effects were not seen: caspase activation was not increased compared to, and morphology did not differ from, the untreated cells. Having established that 1K1 was protective against apoptosis, it was tested within the cryopreservation system.

When 1K1 was used during cryopreservation only, caspase activation was reduced in post-thaw cultures indicating that the apoptotic process is initiated prior to thawing (i.e. throughout the entire cryopreservation protocol). This may be as a result of oxidative (and cold shock) as described above. However, 1K1 was more effective at reducing caspase activation when included both during and after cryopreservation indicating that the apoptotic cascade persists upon thawing. This may be as a result of an injury mechanism similar to reperfusion injury. In terms of ELS recovery, viable cell numbers were significantly improved when 1K1 was included during cryopreservation and per cell functionality was also improved (although this was not significant) making 1K1 a valuable inclusion.

No data regarding the use of (engineered) HGF in liver cell cryopreservation protocols could be found. However, Ryugo et al. found that when recombinant HGF was administered to the rat hearts during hypothermic storage (without cryopreservation), caspase activation was reduced following reperfusion and myocardial function was improved (Ryugo et al. 2004). Only one paper could be found detailing the use of a growth factor in a cryopreservation protocol: Shin et al. found that when ovarian granulosa cells were pre-incubated with vascular endothelial growth factor before cryopreservation, apoptosis in post-thaw cultures was reduced and viability of cells improved (Shin et al. 2006), similar to the results from ELS here.

A more common approach to reducing apoptosis following cryopreservation is to administer caspase blockers, such as Z-VAD-FMK or similar. This approach has been successfully applied to porcine hepatocytes (Yagi, Hardin, Valenzuela, Miyoshi, Gores, & Nyberg 2001), human haematopoietic stem cells (Stroh et al. 2002), mice ovarian tissue (Zhang et al. 2009) and porcine embryos (Men et al. 2006) and universally resulted in reduced caspase activation and improved viability in post-thaw cultures. There was one study using sperm cells where no improvement was shown using Z-VAD-FMK and the authors suggest that the dose used may have been incorrect or that the wrong caspases had been blocked (Men et al. 2006). More recently, Rho-associated kinase (ROCK) has been targeted to limit apoptosis. ROCK is activated downstream of caspase 3 and, once activated, is involved in the completion of apoptosis. ROCK inhibitors have been shown to reduce apoptosis and improve recovery of cryopreserved human stem cells (Gauthaman et al. 2010; Martin-Ibanez et al. 2008). These results confirm that reducing caspase activation following cryopreservation (here using using 1K1) is a valid approach to improving post-thaw recovery.

1K1 also reduced necrosis in post-thaw cultures but only at 3 hours post-warming. This is a somewhat odd result but may result from 1K1's inability to truly reduce necrosis, but simply delay it. Necrosis probably results from ice formation that physically disrupts cell membrane integrity and so altering cell signalling in ELS may be too subtle an approach to be useful in preventing necrosis following cryopreservation.

Finally, as HGF is known to stimulate cell growth, it could be concluded that the improved cell number seen after cryopreservation with 1K1 is simply because ELS growth was stimulated in post-thaw culture and not because it protects against apoptosis. However, the caspase data indicated that it does protect and when ELS were cultured with 1K1 over an extended time period, no increase in cell proliferation was observed. This is useful as stimulation of ELS growth could result in decreased function (as per cell function typically falls with proliferation) or escape of HepG2 cells from the alginate beads if they were to outgrow their scaffold. In the final BAL, a filter system will be in place to prevent transfer of cellular material and other debris back to the patient, but if 1K1 did trigger proliferation, this would present an unnecessary risk.

By using cholesterol and antioxidants and 1K1, ELS recovery is much improved following cryopreservation cf. the starting protocol (see results from Chapter 3). However, all of this work has been completed in small volumes (250µl ELS). As ELS

will comprise the cellular component of a BAL intended for treatment of acute liver failure in adults, this protocol will need to be applied to much larger volumes of ELS, which is the subject of the next chapter.

## 4.6. Conclusions

Encapsulated liver cell spheroids (ELS) suffer from the effects of both apoptosis and necrosis following cryopreservation, which results in losses in viability, cell number and function.

Oxidative stress results after both hypothermic storage and cryopreservation but may be ameliorated using antioxidants, resulting in decreased reactive oxygen species production, decreased oxidative damage and improved ELS recovery.

Apoptosis can be limited using an engineered version of hepatocyte growth factor, 1K1, to result in increased levels of ELS recovery in post-thaw cultures.

This improved protocol will need to be applied to larger volumes of ELS, as would be required to treat an adult patient suffering from acute liver failure.

# CHAPTER 5

# Scale-Up

## 5.1. Introduction

The ultimate aim of this thesis was to develop and optimise a cryopreservation protocol for encapsulated liver cell spheroids (ELS) for use within a bioartificial liver (BAL). In order to treat an adult patient with acute liver failure (ALF), it is estimated that approximately $7x10^{10}$ cells would be required (Coward et al. 2005). This is approximately one third of the cells contained within an adult liver and approximately the same number of cells would remain after a partial hepatectomy from a living donor (i.e. a sufficient number for survival).

### 5.1.1. Encapsulated liver cell spheroid cell density

Up until this point, ELS have been produced using the Inotech syringe pump system and then cultured in static culture conditions in modified alpha-MEM supplemented with 10% fetal calf serum (FCS). However, for the final BAL, ELS will be produced using the JetCutter system and cultured under optimised conditions that will result in increased cell density in order to achieve the required $7x10^{10}$ cells. These optimised culture conditions (that have been developed during the period of the work described here) include culture in a fluidised bed bioreactor (FBB) in modified alpha-MEM supplemented with 10% fresh frozen plasma (FFP), with amino acid supplementation with systems in place to regulate pH and oxygen supply (both static culture and optimised FBB culture methods are more fully described in Chapter 2). These optimised culture conditions can results in ELS cell densities of up to $5x10^7$ cells per ml alginate, considerably more than that of ELS used in experiments so far in this thesis.

As described in Chapter 3, one of the main issues complicating cryopreservation of ELS is that cells are inter-connected which means that cells are more prone to intracellular ice formation (IIF). Chance of IIF is increased in ELS as cellular dehydration is harder to achieve and intracellular ice propagation (IIP) can occur, all of which make cell death in ELS more likely compared to single cells. However, what is unknown is how much of an impact cell density has in terms of ELS recovery. It could be expected that recovery would be worse with increasing cell density based on previously published

literature that highlight the challenges (Balasubramanian et al. 2006) but by how much remains undetermined. It is also conceivable that the different encapsulation and culture systems may alter cell-cell contact formation and the effect of this on cryopreservation success should be elucidated.

### 5.1.2. Cryopreservation volume

Large volumes of ELS (in the region of 1-2 litres) will likely be required to treat an adult with ALF. Of course, this volume may alter depending on the ELS cell density achieved, but cryopreserving ELS in aliquots of 0.25ml is definitely impractical for the final BAL. In Chapter 3, it was demonstrated that temperature control during cooling was important for successful cryopreservation of ELS. Fortunately, ELS are optimally cryopreserved using slow cooling rates and it is easier to apply these to large volumes than rapid cooling rates. Moreover, at increased cell densities, slower cooling rates may prove preferential as these would permit more time for dehydration to occur. However, ELS also require rapid thawing which presents a significant challenge.

Cryopreservation of blood cells in volumes of approximately 100ml has previously been performed in specially-designed plastic bags clamped using an aluminium cassette to limit the thickness (Sputtek et al. 2011). This approach would seem likely to succeed but would require more than one bag to be cryopreserved and ELS pooled before ALF could be treated. It would be preferential for logistical and regulatory issues if cryopreservation of ELS could be performed within the vessel that would eventually be the BAL (i.e. the culture chamber containing the fluidised bed of ELS).

However, this would be more complex as not only would it be a larger volume, the dimensions would be dictated by criteria that were not optimal for cryopreservation (e.g. the vessel would have to be cylindrical, with a diameter of at least 15cm). Heat transfer within such a large vessel would be far more complex and application of an accurate and even cooling rate difficult to achieve. Moreover, achieving rapid thawing would be extremely challenging.

## 5.2. Aims

The first aim of this Chapter was to determine how the ELS cell density affects recovery following cryopreservation and attempt to characterise the differences (if any) between ELS produced and cultured using the two different methodologies.

The second aim was to develop a method of cryopreserving ELS in larger volumes whilst still achieving comparable ELS recovery to ELS cryopreserved in 0.25ml volumes in 1.8ml cryovials.

## 5.3. Methods

### 5.3.1. Cryopreservation of ELS at different cell densities

Materials

ELS in culture

15% DMSO in UW solution (CPA media)

Cholesterol

Kryo10 CRF

1.8ml cryovials

Method

ELS were removed from culture on different days out to day 11. By doing so, different cell densities were achieved. For example, if cells were removed on day 0 or 1, only single encapsulated cells were present. However, if ELS were removed from culture later than this, proliferation had begun and spheroids formed, see Figure 5-1 below. Cell densities of ELS used for these experiments will be indicated in the Results section of this Chapter.

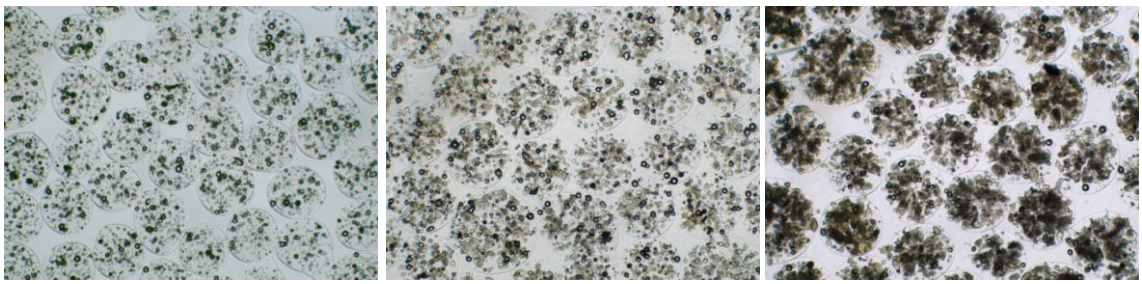

**Figure 5-1. Different ELS cell densities.**

**ELS after 0 (left), 5 (middle), and 8 (right) days of culture after encapsulation. Phase images, original magnification x4.**

ELS were then cryopreserved as in Chapter 2, section 2.9.2. Recovery of ELS was assessed 24 hours later (as described in Chapter 2) and compared to an unfrozen control at equivalent time point. When ELS were removed from FBB culture, they were not returned to FBB culture following cryopreservation as this was impractical but instead were cultured in a rotating cell culture system (RCCS) described in Chapter 2, section 2.2.4.2. Results are expressed as fold change compared to the unfrozen control.

### 5.3.2. Cryopreservation of cell-dense ELS at slower cooling rates

As it is unlikely that cooling at -2°C/minute will be achieved using large volumes of ELS, cell-dense ELS (i.e. ELS cultured in the FBB that contained at least $20x10^6$ cells per ml) were cooled at a slower cooling rate to establish if/how detrimental this was to ELS.

Method

ELS were cryopreserved using the standard method (with cholesterol) as described in Chapter 2, section 2.9.2 except that instead of the main cooling ramp set to cool at 2°C/minute, it was modified to cool at 0.3°C/minute.

### 5.3.3. Cryopreservation of ELS with different volumes of cryoprotectant medium

The standard cryopreservation protocol used throughout this thesis uses an alginate bead to CPA media ratio of 1:4. However, it would be useful if this ratio could be reduced as this would mean that a greater volume of ELS relative to CPA media could be frozen with each run.

Materials

ELS

15% DMSO in UW solution containing 1.1mg/ml cholesterol (CPA media)

1.8ml cryovials

Kryo10 CRF

Method

ELS were mixed with ice-cold CPA media at a ratio of 1:4 (standard) and pipetted into cryovials in different volumes. The alginate beads were allowed to settle (on ice) before CPA media was removed in different volumes. The volumes used to achieve different ratios of alginate beads to CPA media volumes are given in Table 5-1 below.

**Table 5-1. Method to achieve different starting volume ratios of alginate beads to cryoprotectant media.**

| Starting ELS:CPA media ratio | Volume into cryovial (µl) | Volume CPA media removed (µl) | Final ELS:CPA media ratio |
|---|---|---|---|
| 1:4 | 625 | 0 | 1:4 |
| 1:4 | 1562.5 | 937.5 | 1:1 |
| 1:4 | 3125 | 2500 | 1:0 |

As 3.125ml will not fit in a 1.8ml cryovial (to achieve 1:0), this was performed in 2 stages.

### 5.3.4. Cryopreservation in cryobags

Cryopreservation of ELS was tested in 250ml cryobags clamped using an aluminium cassette as an intermediate step up in cryopreservation volume. Although the total volume of the bag was 250ml, the recommended fill volume was 100ml to allow for expansion of the volume once frozen and compression of the bag by the aluminium cassette (which limited bag thickness to 3mm).

Materials

ELS

UW solution

24% DMSO in UW containing 2.2mg/ml cholesterol crystals (CPA media)

50ml syringe

Alumnium cassette (Baxter CASS350)

Freezing bag (Baxter Cryocyte R4R9953)

Heatsealer

Kryo 10 CRF

Method

ELS were washed with UW solution once before being mixed well with CPA media at a ratio of 1:1 on ice. ELS were then loaded into a 50ml syringe and transferred into the bag on ice via the inlet port. This process was repeated to achieve a final volume of approximately 80ml inside the bag. The bag was carefully loaded into the aluminium cassette ensuring the inlet port was held high (to prevent spillage). Once the aluminium cassette was closed, the inlet port was closed using a heatsealer. The bag was then transferred into the CRF chamber and ELS cooled using a non-linear cooling profile (Diener et al. 1993).

#### 5.3.4.1. Measuring temperature profiles in cryobags during cooling and warming

Materials

Type K thermocouple

Picologger

Autoclave tape

Method

Thermocouples were placed at various locations (see Figure 5-2) on the external edge of the aluminium cassette and were held in place using autoclave tape. Thermocouples were also threaded into the bag via the inlet port. As the thermocouples were very thin, the inlet port could still be closed even when thermocouples were there.

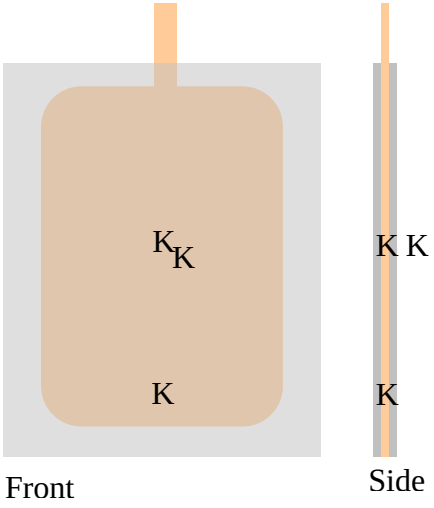


**Figure 5-2. Experimental setup for cryobag cryopreservation and measuring temperature profiles.**

**Pink indicates cryopreservation bag containing ELS. Grey indicates aluminium cassette. K indicates location of thermocouple. Front (left) and side (right) views are shown.**

5.3.4.2. Comparison between cryopreservation in cryobags and cryovials

Method

To compare cryopreservation in bags and cryopreservation in cryovials, ELS were cryopreserved in both at the same time using the same methodologies, as described in section 5.3.1 above. The only difference was that ELS were cryopreserved in 80ml volumes in bags and in 1ml volumes in cryovials. Recoveries of the two cryopreserved cohorts were compared to each other, and to unfrozen ELS at equivalent time points to 24 hours post-warming, using the viability, cell number and hepato-specific protein synthesis and secretion assays (described in Chapter 2).

### 5.3.5. Cryopreservation in culture chamber

If ELS could be cryopreserved in a disposable culture chamber, this would truly provide an off-the-shelf solution as originally intended and so cryopreservation in a small culture chamber was attempted.

5.3.5.1. Cooling of culture chamber

Cooling of the culture chamber (described in Chapter 2, section 2.2.4.1) was achieved using the Kryo10 CRF and the protocol was largely similar to those previously described throughout this thesis, except that attempts were made to reduce the mass to be cryopreserved as much as possible, to ease heat transfer.

Materials

UW solution

24% DMSO with 2.2mg/ml cholesterol (CPA media)

ELS

500ml pyrex beaker (baked at 180°C for 3 hours)

1mm thick silicon sheeting (Altec 03-65-4609)

-80°C freezer

Method

Prior to cryopreservation, the bottom of the chamber was blocked off using silicon sheeting and the whole chamber was cooled on ice. ELS were washed once with iced UW solution and mixed with CPA media at a ratio of 1:1 in a sterile beaker. 500ml of this was then poured into the culture chamber. The whole chamber was then transferred to the Kryo10 CRF and cooled using a non-linear cooling profile (Diener et al. 1993). Unfortunately, the chamber could not be stored in the vapour phase of liquid nitrogen as the facilities to store such a large item were not available and so the chamber was removed once it had reached -80°C and transferred to a -80°C freezer for overnight storage before thawing.

5.3.5.2. Warming of culture chamber

*5.3.5.2.1. Standard warming of culture chamber*

To directly compare warming in the culture chamber and cryovials, the culture chamber was warmed using an identical method to that normally used to warm cryovials.

Method

The culture chamber was removed from the -80°C freezer and placed into a 37°C water bath. The culture chamber was shaken gently and occasionally to try to encourage the ice to break into smaller pieces to increase rate of melting. Once all visible ice had melted, ELS were removed and returned to culture for assessment of recovery, after 24 hours.

*5.3.5.2.2. Improved warming of culture chamber*

ELS are optimally thawed using rapid warming rates. In an attempt to increase the warming rate, the protocol was modified slightly.

Materials

50% v/v ethylene glycol in water

Copper tubing

Silicon sheeting

Alginate beads with (for testing ELS recovery) and without cells (for trial run)

37°C water bath

60°C water bath

1L Schott bottles (with modified lids to allow for entry and exit of warmed liquid via silicon tubing)

Silicon tubing

Watson-marlow pump (400ml/minute)

Method

The improved warming protocol was identical to that described above except that in addition to warming from the external 37°C water bath, warming from inside the cell mass was also attempted to determine whether or not it was possible to improve ELS recovery if rapid warming rates were used following cryopreservation in the culture chamber in large volumes. A prototype coil was made to test this. Warming from inside the frozen mass was achieved by pumping an ethylene glycol solution through a coil of made-for-purpose copper tubing. The copper tubing was kindly made to my design by Mr. Chandra Mistry in the Dept. of Medical Engineering, UCL. For clarity, the experimental set-up of the improved warming protocol is shown in Figure 5-3.

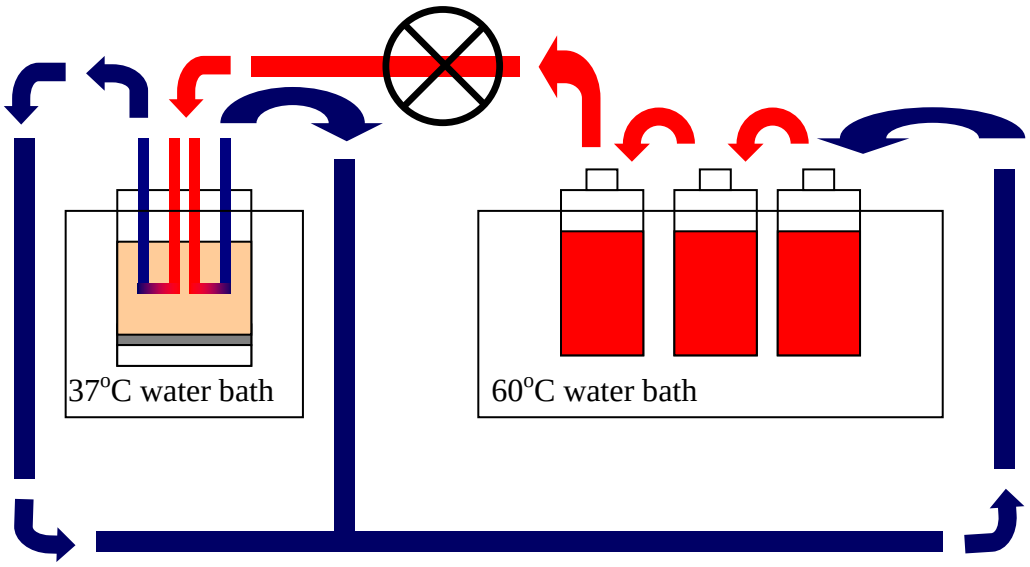


**Figure 5-3. Experimental set-up for improved culture chamber warming.**

**The entire culture chamber was placed into a 37°C water bath. 3L of 50% v/v ethylene glycol solution were warmed to 60°C using a 60°C water bath. The ethylene glycol solution was pumped into the copper tubes into the centre of the culture chamber, around the circuit, out of the culture chamber and back into the bottles in the 60°C water bath at 400ml/minute using a pump. This cycle continued until the ice in the culture chamber had melted. Red indicates warm liquid. Blue indicates cold liquid. Pink represents ice and alginate beads. Grey indicates silicon sheeting.**

#### 5.3.5.3. Measuring temperature profiles in culture chamber during cooling and warming

Materials

Type K thermocouple

Picologger

Autoclave tape

Method

Thermocouples were placed in the culture chamber and (where applicable in the bottles containing ethylene glycol in the 60°C waterbath).

## 5.4. Results

### 5.4.1. Effect of ELS cell density on recovery following warming

As an initial investigation, Inotech-encapsulated ELS were cryopreserved on days 1 and 10 following encapsulation. When ELS were cryopreserved on day 1, viability, viable cell numbers and total function were unaffected, indicating a good overall recovery. However, when ELS were cryopreserved on day 10, viability, viable cell numbers and function were all significantly reduced by 10%, 40% and 30% respectively. This correlated with the much higher cell densities at day 10. These results are summarised in Table 5-2 below.

**Table 5-2. Effect of ELS cell density on recovery following cryopreservation**
**Values given in table are means of n=5 +/- SD from a single experiment.**

| | Day 1 | Day 10 |
|---|---|---|
| Unfrozen viability (%) | 99.2 +/- 0.2 | 98.5 +/- 0.2 |
| Cryopreserved viability (%) | 99.2 +/- 0.3 | 87.1 +/- 3.4 |
| Fold change cf. unfrozen | 1 | 0.9 |
| Statistics | NS | $p<0.05$ |
| Unfrozen viable cell number ($10^6$ nuclei/ml) | 3.08 +/- 0.16 | 13.1 +/- 0.7 |
| Cryopreserved viable cell number ($10^6$ nuclei/ml) | 2.66 +/- 0.28 | 8.53 +/- 0.6 |
| Fold change cf. unfrozen | 0.9 | 0.6 |
| Statistics | NS | $p<0.005$ |
| Unfrozen albumin production (µg/well/24h) | 20.1 +/- 1.2 | 81.0 +/- 2.2 |
| Cryopreserved albumin production (µg/well/24h) | 16.9 +/- 1.9 | 55.0 +/- 2.9 |
| Fold change cf. unfrozen | 0.8 | 0.7 |
| Statistics | NS | $p<0.01$ |

When ELS were cryopreserved over a range of cell densities, a strong negative correlation between cell density and recovery in terms of viability (see Figure 5-4

below) and viable cell number (Figure 5-5) was observed. This was true for ELS encapsulated using both the Inotech and cultured in static conditions ($r^2$=0.9801) and for ELS encapsulated using the JetCutter and cultured in the FBB ($r^2$=0.8845) (see Figure 5-5).

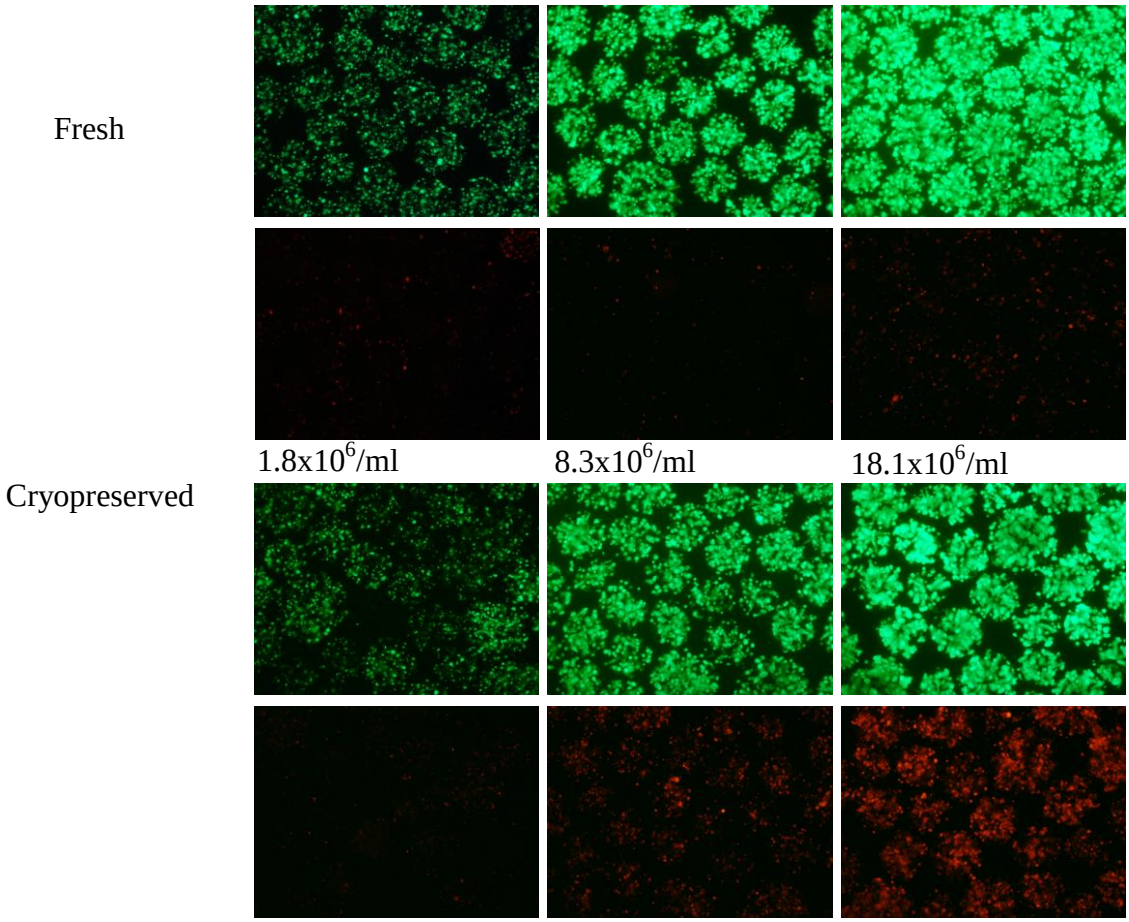


**Figure 5-4. Effect of ELS cell density on ELS viability in post-warming cultures.**

**ELS were cryopreserved using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol at different cell densities (as indicated). ELS were thawed rapidly (37°C waterbath) and viability assessed using FDA (green/live) and PI (red/dead) stains. Cryopreserved ELS (bottom) were compared to fresh ELS (top) at equivalent time point. Images are representative of 4 further repeats. 64ms exposure for FDA, 250ms exposure for PI. Original magnification x4.**

The latter also showed slightly reduced recovery following cryopreservation when compared to the former (i.e. JetCutter-encapsulated were more affected by cryopreservation protocol than Inotech-encapsulated) (Figure 5-5).

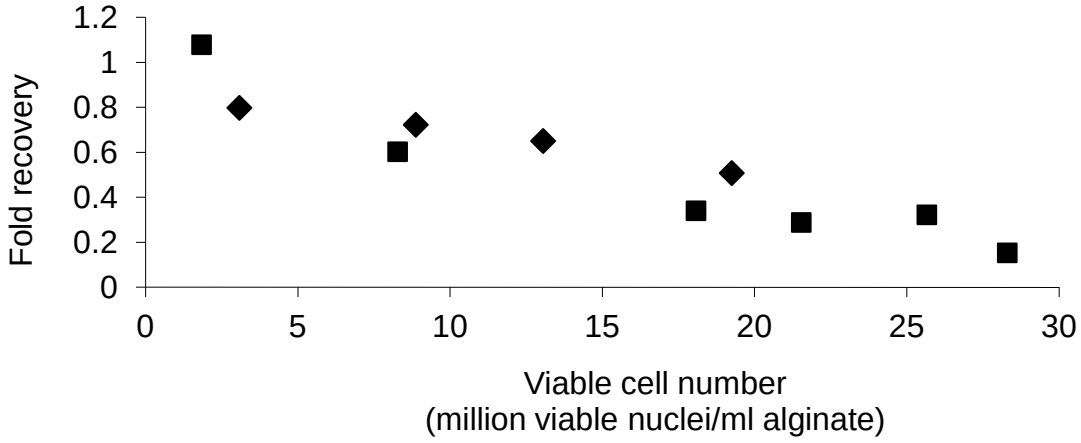


**Figure 5-5. Effect of ELS cell density on ELS viable cell number in post-warming cultures.**

**ELS were encapsulated either using the JetCutter (■) or the Inotech (♦) and cryopreserved at different cell densities, as indicated on the x axis. Results are expressed as fold change compared to unfrozen ELS at equivalent time point.**

### 5.4.2. Effect of slower cooling rates for cell-dense ELS

5.4.2.1. Viable cell number

Viable cell numbers were slightly higher for cell-dense ELS (i.e. ELS cultured in the FBB that contained at least $20x10^6$ cells per ml) cryopreserved at 0.3°C/min compared to those cryopreserved at 2°C/minute at both 24 and 48 hours post-warming, although this was only statistically significant at the later time point (Figure 5-6).

5.4.2.2. Function

Function was also improved for cell-dense ELS that were cryopreserved at 0.3°C/min when compared to those cryopreserved at 2°C/minute at both time points. However, this was more apparent and only statistically significant at 24 hours post-warming (Figure 5-7).

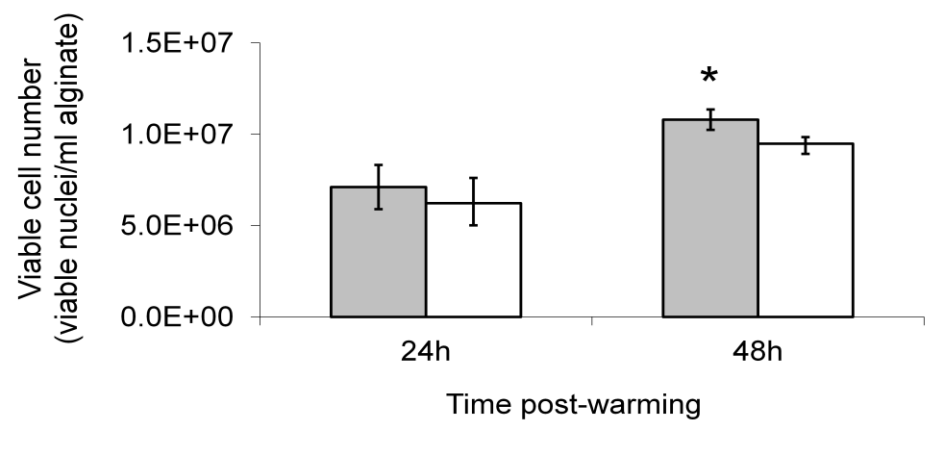


**Figure 5-6. Effect of slow cooling on cell-dense ELS viable cell number in post-warming cultures.**

**Cell-dense ELS were cooled at either 0.3°C/minute (grey bars) or 2°C/minute (white bars) between -8°C and -60°C before more rapid cooling to -160°C in 12% DMSO/UW with cholesterol. ELS were warmed rapidly (37°C waterbath) and viable cell numbers quantified at 24 and 48 hours post-warming. Data are means of n=5 RCCS +/- SD from a single experiment. *p<0.05 compared to ELS cooled at 2°C/minute at the same time point.**

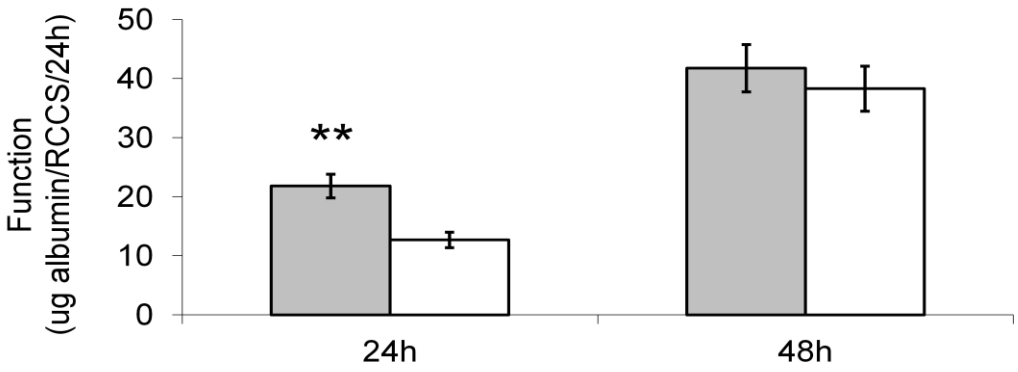


**Figure 5-7. Effect of slow cooling on cell-dense ELS function in post-warming cultures.**

**Cell-dense ELS were cooled at either 0.3°C/minute (grey bars) or 2°C/minute (white bars) between -8°C and -60°C before more rapid cooling to -160°C in 12% DMSO/UW with cholesterol. ELS were warmed rapidly (37°C waterbath) and albumin production quantified at 24 and 48 hours post-warming. Data are means of n=5 RCCS +/- SD from a single experiment. **p<0.01 compared to ELS cooled at 2°C/minute at the same time point.**

### 5.4.3. Effect of different ELS:CPA ratio on ELS recovery following warming

5.4.3.1. Viable cell number

Viable cell numbers of all 3 cryopreserved cohorts were reduced when compared to the unfrozen control, significantly for the 2 conditions where the least CPA media was used. Moreover, ELS cryopreserved at an ELS to CPA ratio of 1:0, showed

significantly reduced viable cell numbers in post-thaw culture compared to those cryopreserved at an ELS to CPA ratio of 1:4 (Figure 5-8).

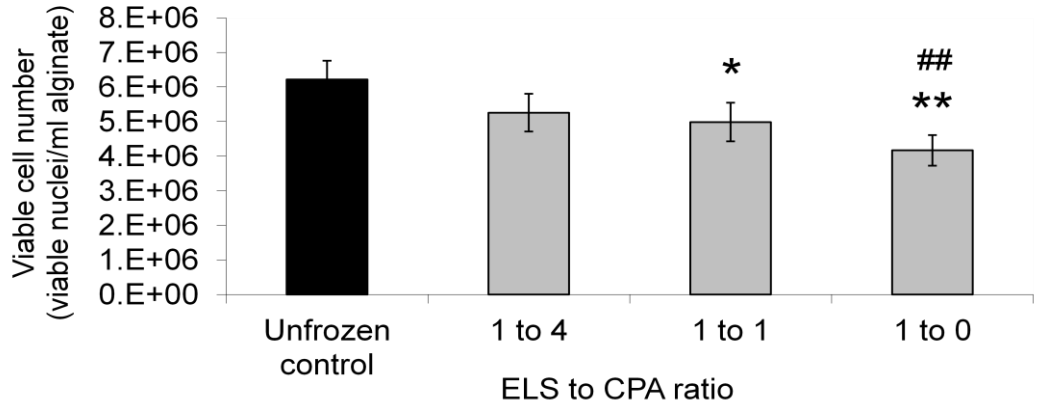


**Figure 5-8. Effect of ELS to CPA ratio on viable cell numbers in post-warming cultures.**

**ELS were cryopreserved using a multistep cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol at different ratios of ELS to CPA as indicated on the x axis. ELS were thawed rapidly (37°C waterbath) and compared to unfrozen ELS at equivalent time point. Data are means of n=5 wells +/- SD from a single experiment. *p<0.05, **p<0.01 compared to unfrozen control ELS. ##p<0.01 compared to ELS cryopreserved at a ratio of 1 to 4.**

### 5.4.3.2. Function

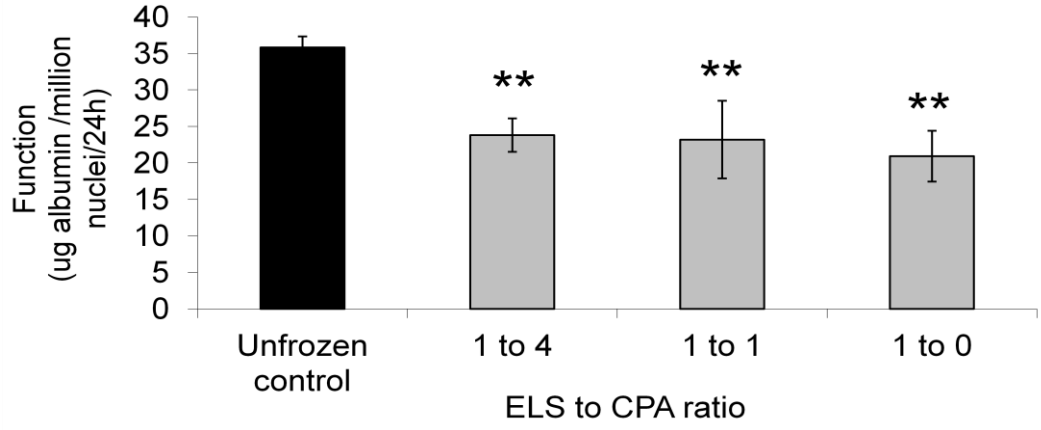


**Figure 5-9. Effect of ELS to CPA ratio on function in post-warming cultures.**

**ELS were cryopreserved using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol at different ratios of ELS to CPA as indicated on the x axis. ELS were thawed rapidly (37°C waterbath) and compared to unfrozen ELS at equivalent time point. Data is normalised to viable cell nuclei. Data are means of n=5 wells +/- SD from a single experiment. **p<0.01 compared to unfrozen control ELS.**

Per cell function of all cryopreserved cohorts was reduced when compared to unfrozen ELS. However, there was no difference in function between any of the cryopreserved cohorts.

### 5.4.4. Cryopreservation in cryobags

5.4.4.1. Temperature profiles

*5.4.4.1.1. Cooling*

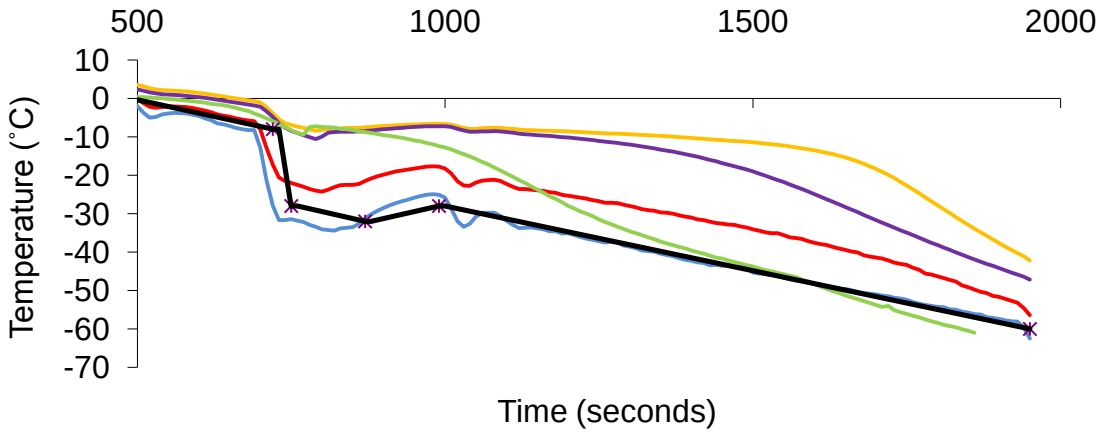


**Figure 5-10. Comparison of time-temperature profiles for cryovials and cryobags during cooling.**

**ELS were cooled using a multi-step cooling profile (solid black line) (Diener et al. 1993). Traces show temperature of CRF chamber (blue), external edge of bag cassette (red), edge inside of bag (purple), middle inside of bag (orange) and inside the cryovial (green).**

The Kryo 10 CRF was able to complete the programmed cooling profile, with only a little over-shoot apparent after the shock-cooling step and slight modulation thereafter. Cryobags and cryovials were cooled in the same run to allow comparison of the temperature profiles. The cryovial initially cooled with the chamber, the sample supercooled slightly by approximately 2°C, latent heat was released following nucleation but after 1000 seconds, sample temperature fell to reach that of the chamber. The external side of the bag cassette cooled in time with the chamber with only a slight delay but did not reach the low temperatures achieved by the chamber, instead remaining approximately 10°C warmer. At both locations within the bag (i.e. centre and edge) supercooling of approximately 2.5°C was measured. Again, latent heat of crystallisation was released following ice crystallisation. After this, bag sample temperature did fall towards that of the CRF chamber but took longer than the cryovial sample (approximately 1500 seconds).

*5.4.4.1.2. Warming*

Warming profiles of cryovials and cryopreservation bags were very similar when thawed using a 37°C water bath. Average thawing rate for both was approximately +80°C/minute and both were thawed within 2 minutes. Both containers showed an exponential warming profile (i.e. rapid warming initially, followed by slower warming at temperatures closer to the melting point).

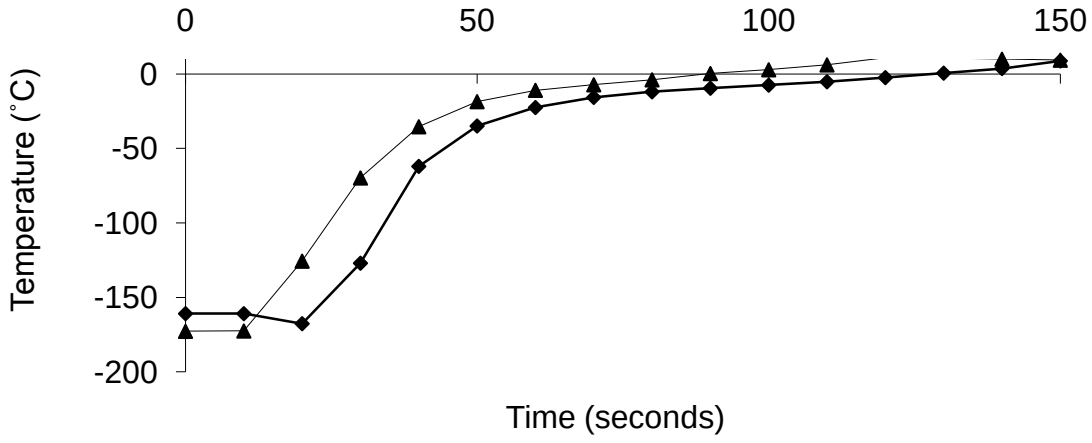


**Figure 5-11. Comparison of time-temperature profiles for cryovials and cryobags during warming. ELS in cryovials (♦) and cryopreservation bags (■) were warmed in a 37°C water bath.**

5.4.4.2. Recovery

*5.4.4.2.1. Viability*

Viability of both cryopreserved cohorts was approximately 65%. The viabilities of ELS cryopreserved in either cryovials or cryopreservation bag were significantly lower than the unfrozen control (92%) ($p<0.005$) but were not statistically different from each other ($p=0.77$), see Figure 5-12. This recovery is slightly lower than what could have been expected if compared to results from Chapter 3, but this is due to the increased cell density of ELS used in this experiment.

*5.1.1.1.1 Viable cell number*

Viable cell numbers of both cryopreserved cohorts of ELS were significantly reduced when compared to unfrozen control ELS by approximately 50% ($p<0.005$) but were not statistically different from each other ($p=0.78$), see Figure 5-13.

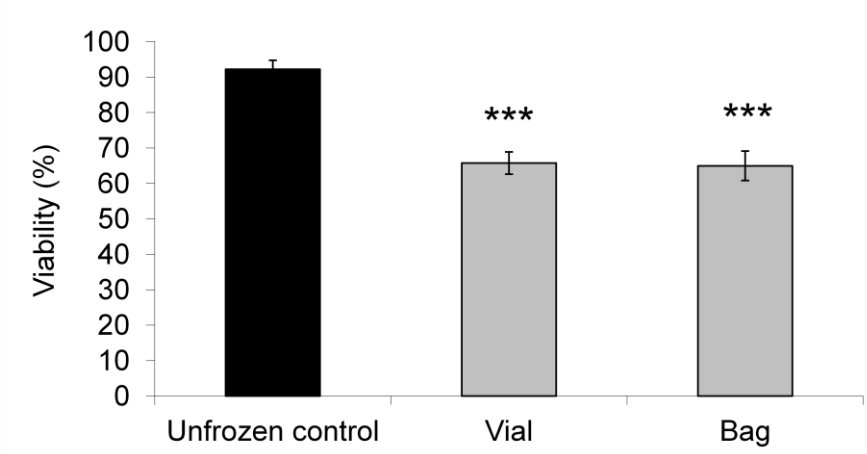


**Figure 5-12. Comparison of ELS cryopreserved in cryovial or cryobag – viability.**

**ELS of the same batch were cryopreserved using identical cooling profile (Diener et al. 1993) at the same time in 12% DMSO/UW with cholesterol. Both were thawed rapidly using a 37°C waterbath and viability compared at 24 hours post-warming and compared to unfrozen control ELS at equivalent time point. Data are n=5 fields +/- SD from a single experiment. ***p<0.005 compared to the unfrozen control.**

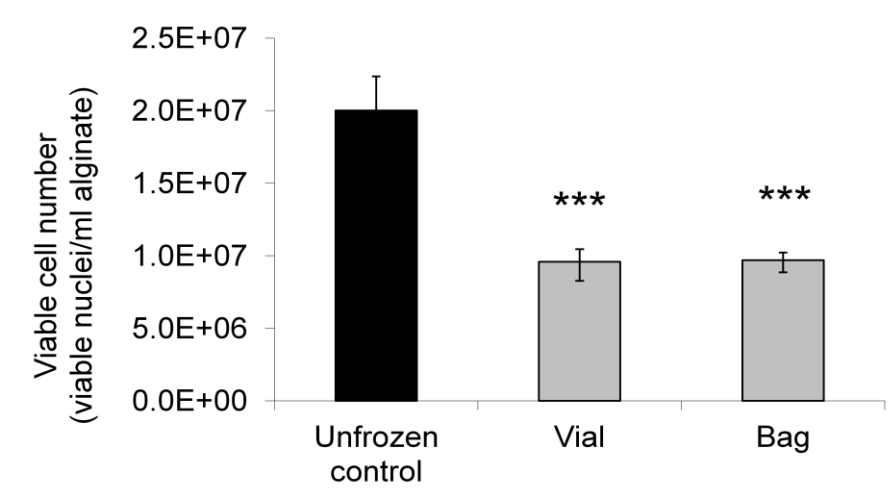


**Figure 5-13. Comparison of ELS cryopreserved in cryovial or cryobag – viable cell numbers.**

**ELS of the same batch were cryopreserved using identical cooling profile (Diener et al. 1993) at the same time in 12% DMSO/UW with cholesterol. Both were thawed rapidly using a 37°C waterbath and viability compared at 24 hours post-warming and compared to unfrozen control ELS at equivalent time point. Data are n=5 wells +/- SD from a single experiment. ***p<0.005 compared to the unfrozen control.**

### 5.4.5. Cryopreservation in culture chamber

5.4.5.1. Temperature profiles

*5.4.5.1.1. Cooling*

ELS cooled in cryovials were cooled to -60°C in approximately 20 minutes, in line with the programmed cooling profile. Supercooling of approximately 2°C was measured, in agreement with previous data. However, ELS cooled in the culture chamber took approximately 90 minutes to reach -60°C, thus achieving an average cooling rate of 0.67°C/min (far slower than the programmed cooling rate) with no evidence of supercooling *per se* although there was a slight "flattening" in the sample cooling profile around -6°C. Moreover, the effective cooling rate was not linear, ELS were cooled more rapidly initially than later.

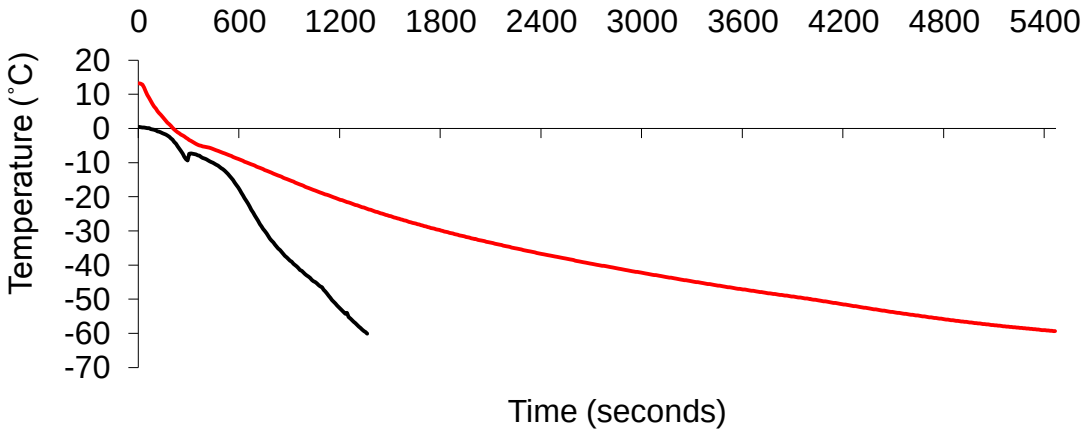


**Figure 5-14. Comparison of time-temperature profiles for cryovials and culture chamber during cooling.**

**ELS were cooled using the Terry profile. Traces show temperature of cryovial (black line) and the culture chamber (red line).**

*5.4.5.1.2. Warming*

Alginate beads were warmed using a 37°C waterbath. Those cryopreserved in cryovials were thawed in approximately 2 minutes. However, ELS cryopreserved in the culture chamber took approximately 2 hours to thaw, considerably longer than the cryovials. A bi-phasic warming profile was apparent, with more rapid warming rates achieved initially. Using the modified culture chamber, thawing was complete in approximately 10 minutes and was more linear. The time-temperature profiles for all 3 are shown in Figure 5-15.

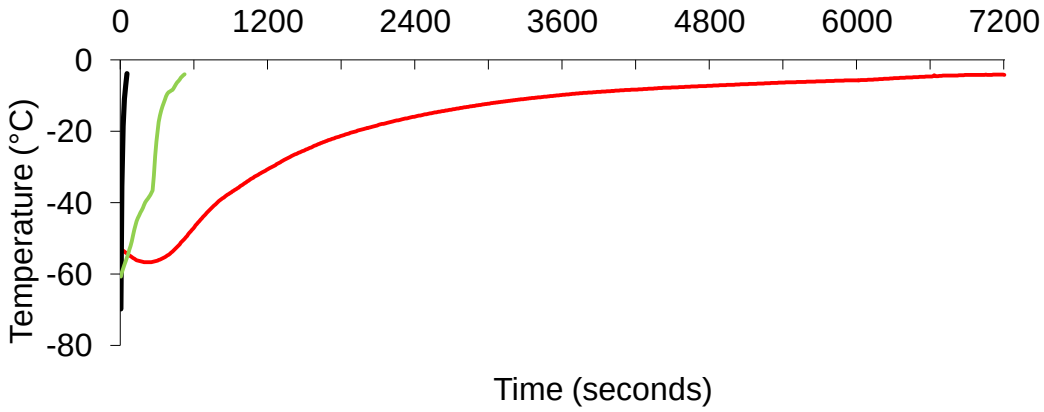


**Figure 5-15. Comparison of warming profiles between cryovial, culture chamber and modified culture chamber.**

**Sample temperatures until thawing (i.e. approximately -3°C) are shown. Time-temperature traces are from cryovials (black), culture chamber (red) and modified culture chamber (green) from a single experiment.**

The temperature of the ethylene glycol solution in the 60°C water bath fell gradually over time taken for the modified culture chamber to thaw by approximately 30°C, with a rapid decline in temperature at approximately 450 seconds. This was an indication of the heat transfer required to rewarm the larger culture chamber.

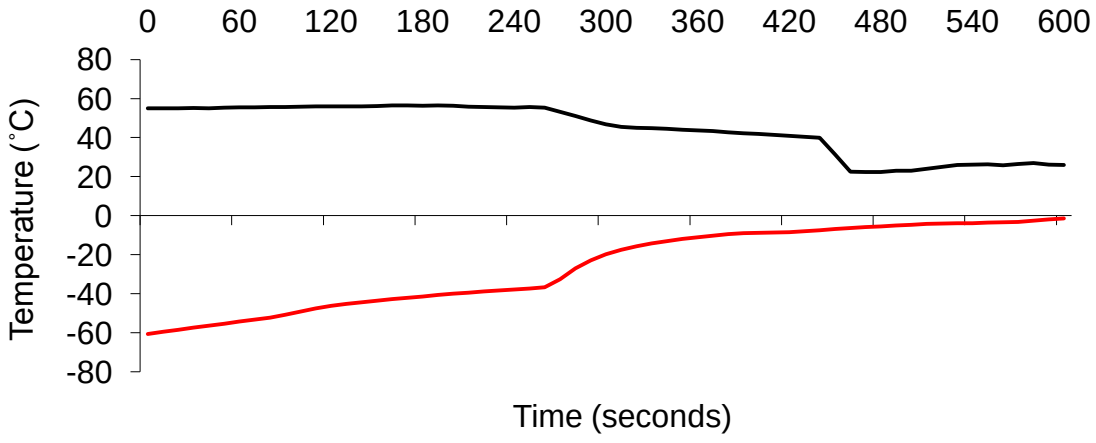


**Figure 5-16. Time-temperature profiles during modified culture chamber warming.**

**Time-temperature traces are from modified culture chamber (red) and ethylene glycol solution (black) from a single experiment.**

5.4.5.2. ELS recovery following cryopreservation in culture chamber using standard thawing methodology

*5.4.5.2.1. Viability*

The unfrozen control maintained a good viability throughout (>98%). ELS cryopreserved in the cryovial showed high levels of PI staining relative to FDA after 24h (38 +/- 12% viable) but this PI staining fell by 48 hours (41 +/- 21% viable). ELS cryopreserved in the cryobag showed similar recovery to ELS cryopreserved in the cryovial in agreement with previous findings with quantified viabilities of 47 +/- 3% viable and 71 +/- 20% viable at 24 and 48 hours respectively. However, ELS cryopreserved in the culture chamber showed lower amounts of FDA staining after 24 hours and by 48 hours, very little positive FDA staining was observed. Captured images are shown below in Figure 5-17.

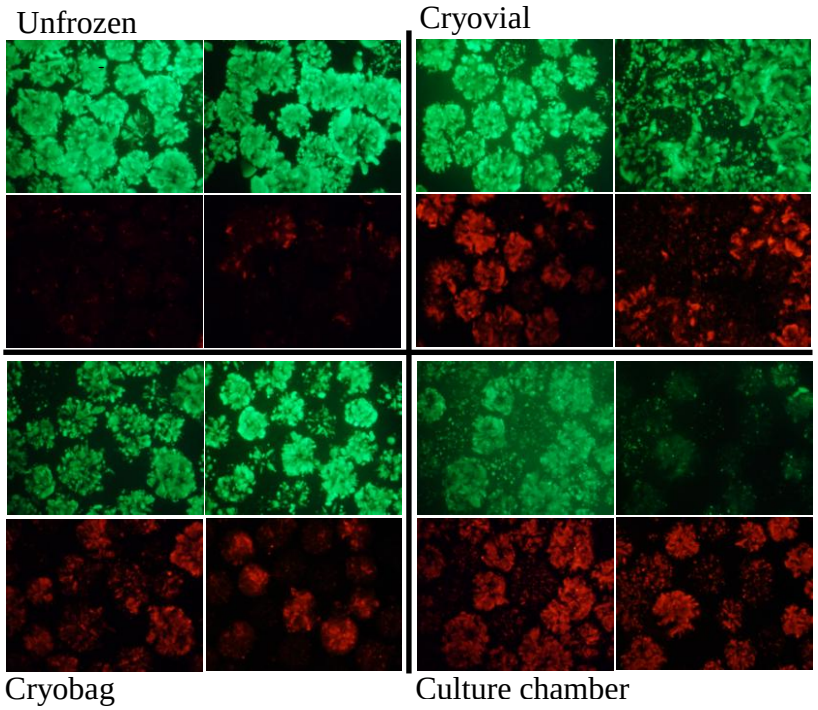


**Figure 5-17. Qualitative assessment of viability in post-warming cultures following cryopreservation and thawing in different vessels.**

**ELS were cryopreserved in either cryovials, cryobags or the culture chamber using a multi-step cooling profile (Diener et al. 1993) in 12% DMSO/UW with cholesterol. ELS were thawed rapidly (37°C waterbath) and compared to an unfrozen control at equivalent time point. ELS were stained with PI (red) and FDA (green) at 24 and 48 hours post-warming (right hand side in each panel). Images are typical of 5 captured fields from a single experiment. FDA 64ms exposure, PI 250ms exposure. Original magnification x4.**

Quantified viabilities for ELS cryopreserved in the culture chamber were 29 +/- 6% at 24h reduced to 4 +/- 2% at 48 hours. Quantitative data is shown in Figure 5-18 below.

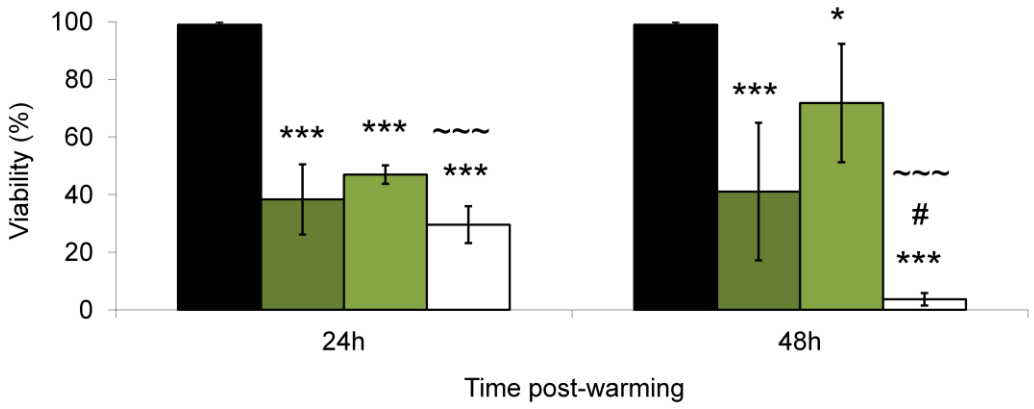


**Figure 5-18. Quantitative assessment of viability in post-warming cultures following cryopreservation and thawing in different vessels.**

**ELS were cryopreserved in either cryovials (dark green bars), cryobags (light green bars) or the culture chamber (white bars) and compared to an unfrozen control (black bars) at equivalent time point. Data are means of n=5 +/-SD from a single experiment. *p<0.05, ***p<0.005 cf. unfrozen control at equivalent time point. #p<0.05 cf. ELS cryopreserved/thawed in cryovial at same time point. ~~~p<0.005 cf. ELS cryopreserved/thawed in cryobag at same time point.**

### 5.4.5.3. ELS recovery following cryopreservation in culture chamber using optimised thawing methodology

Although the optimised thawing methodology did demonstrate efficacy at warming the cryopreserved mass sufficiently quickly (see Figure 5-16), I was unable to demonstrate whether or not this improved ELS recovery as the tubing failed on a run with ELS. This highlights the bio-engineering challenges which need to be addressed in future studies to develop chamber constructions in materials capable of withstanding wide temperature fluctuations during transit to and from cryogenic temperatures.

## 5.5. Discussion

In the previous Chapters, cryopreservation of ELS typically containing no more than $1x10^7$ cells per ml alginate in small volumes of 0.25ml was optimised. However, for the final BAL, cryopreservation of ELS containing larger numbers of cells (i.e. a greater cell density will be required) and, moreover, in larger volumes will be required. This should enable an adult patient suffering from ALF to be effectively treated using a BAL. Therefore, the first aim of this Chapter was to investigate the effect of increasing ELS cell density on recovery following cryopreservation. The second aim of this Chapter was to attempt to develop a method for successful cryopreservation of ELS in larger volumes, ideally within the culture chamber so that a truly off-the-shelf cell therapy could be provided.

To investigate the effect of the cell density of ELS upon recovery following cryopreservation, ELS of different cell densities were cryopreserved and results compared to an unfrozen control at an equivalent time point. Initially, 2 cell densities were selected: $3x10^6$/ml and $13x10^6$/ml. Whilst the lower cell density were successfully cryopreserved (with no statistically significant differences between fresh and cryopreserved ELS), those at the higher cell density were significantly detrimentally affected by the cryopreservation process as assessed using all 3 recovery parameters. When this was investigated over a wider range of cell densities (to a maximum of $30x10^6$/ml) a strong negative correlation between cell density and recovery in post-thaw recovery was observed. This was true for both viability and viable cell number and for both ELS encapsulated using both the Inotech and JetCutter.

As described in more detail in Chapter 3, cryopreservation of single cells and spheroids differs greatly. Inter-connected cells, such as ELS, are more prone to suffer the damaging effects of too rapid cooling rates as they do not have sufficient time to dehydrate, are more likely to form intracellular ice which can then propagate between cells, reducing recovery greatly (Acker et al. 1999; Berger & Uhrik 1996; Ehrhart et al. 2009; Korniski et al. 1999). In terms of this study, day 1 encapsulated HepG2 cells can be considered as almost single cells; single cells are encapsulated on day 0 and proliferation is not immediate as individual cells first anchor themselves to the surrounding alginate matrix. However, beyond this time point, HepG2 cells will continue to proliferate until the culture conditions can no longer support the cell density (above ~ $6x10^7$/ml).

Here, as cell density increased, recovery in post-thaw cultures fell. This could have been expected as when cell density increases, more time may likely be required for dehydration to remove any persisting "freezable" water, simply as water must travel a greater distance from the centre to periphery of the spheroid before exiting. In Chapter 3, for cell densities no greater than $1x10^7$/ml, this was achieved using a nucleator and a cooling rate of 2°C/minute. However, this approach appears to be insufficient for ELS at greater cell densities presumably as this does not allow sufficient time for complete ELS dehydration prior to nucleation.

To support this hypothesis, when ELS were cooled using a slower (than 2°C/min) main cooling ramp of 0.3°C per minute, not only was this not detrimental (as it was to less cell-dense ELS as shown in Chapter 3) a general trend (in terms of viable cell number and function) of slightly improved recovery was observed. An alternative cause of reduced recovery in cell-dense ELS could be that of incomplete penetrating CPA permeation. Although DMSO is considered to be readily permeable, much of the published work focuses on single cell suspensions. CPA transport within tissue or tissue-like ELS will differ and is likely to take longer. For example, permeation of 6.9M DMSO into cartilage plugs (measuring 8mm diameter, 5-10mm thick – much larger than ELS) took up to 4 hours to complete (Mukherjee et al. 2008). In another study, permeation of 1M DMSO into heart valve leaflets at 4°C was not complete even after 6 hours (Lakey et al. 2003). In a third study, permeation of 1.5M DMSO into $2mm^3$ blocks of ovarian tissue was complete sooner after 40 minutes at 4°C (Newton et al. 1998). Although it is difficult to compare these studies to ELS, due to differing cell type and size of respective samples, the incubation step (15 minutes) used for ELS in this study, was shorter than any used in the above studies and so increasing the length of the incubation step may be beneficial. However, this potential benefit will have to be balanced against the potential cytotoxicity of prolonged DMSO exposure.

As a further point of interest, ELS produced using the JetCutter and cultured in the FBB appeared to show slightly reduced recovery when compared to ELS of similar densities produced using the Inotech system and cultured in a static environment. There could be several plausible explanations for this (such as an increased susceptibility of FBB-cultured ELS to removal from such an optimised culture environment) but it could be the formation of structurally different spheroids.

FBB-cultured cells tend to form more spherical spheroids than those cultured in a static environment. Static cultured (Inotech-encapsulated) ELS tend to form thinner, more elliptical spheroids. For ease of interpretation, images of ELS at the same cell density produced by both methods are shown in Figure 5-19 below. This difference in spheroid shape may be as a result of different stresses encountered by the spheroids during culture (FBB-cultured ELS are grown in a shear stress-free environment (Coward et al. 2005)) or the effect of different initial cell seeding densities. JetCutter-encapsulated and FBB-cultured ELS are seeded at a higher cell density than Inotech-encapsulated/static-cultured ELS ($1.75x10^6$/ml vs. $0.5x10^6$/ml) and so perhaps this increased seeding density alters spheroid morphology.

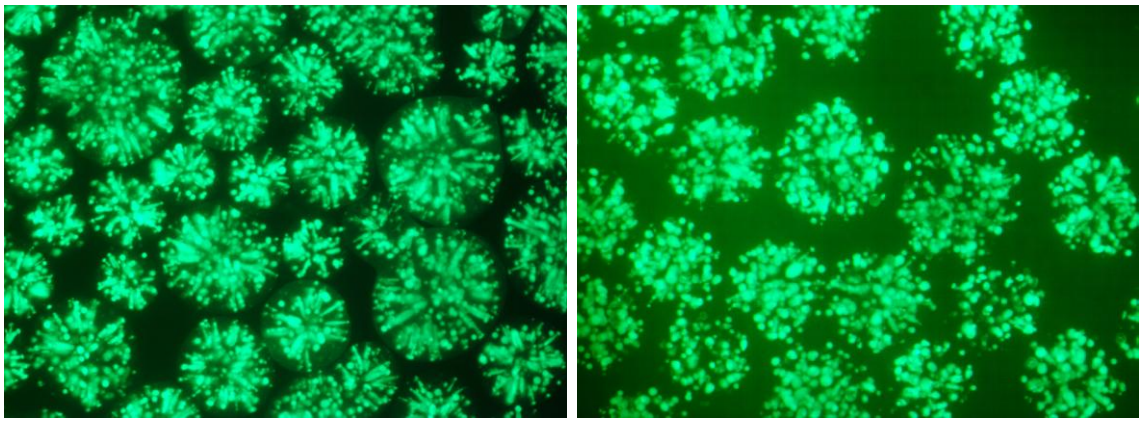

**Figure 5-19. Different spheroid shapes resulting from different methods of encapsulation and culture.**

**Images show FDA staining of ELS both at cell densities of $9x10^6$/ml encapsulated using the Inotech (left) and JetCutter (right). 64ms exposure time. Original magnification x4.**

Whatever the cause, a more spherical spheroid may take longer to dehydrate as there is no short route by which water can exit the spheroid mass. This may account for the slightly decreased recovery of FBB-encapsulated ELS although to confirm or disprove this, a more detailed study would need to be devised. Clearly, the current protocol for ELS cryopreservation is not sufficient for cell-dense ELS. This is probably a result of the more complex water transport characteristics and difficulties in ensuring sufficient dehydration. Strategies to avoid this could include application of slower cooling rate, shortening/removal of the shock cooling step and/or use of different non-penetrating CPAs. Similarly, complete permeation of CPA may be more difficult to achieve in a more spherical spheroid as, again, there is no short route by which it may penetrate to the centre of the spheroid.

The second aim of this Chapter was to attempt to cryopreserve ELS in large volumes. The current protocol utilises a bead to CPA ratio of 1:4. This ratio was initially selected as it enabled easy transfer of ELS (i.e. ELS could be pipetted into cryovials from culture) and moreover, enabled ready mixing of beads with CPA. However, when attempting to upscale the cryopreservation protocol, the excess CPA could be considered disadvantageous as it presents more of a challenge to cooling (i.e. it is far harder to accurately and evenly cool a larger mass than a small one). Once the beads have been transferred to the cryopreservation vessel, the presence of the excess CPA is not required and so different bead to CPA ratios were trialled, with the aim of reducing CPA volume.

Reducing the CPA volume to 1:1 (e.g. 1ml CPA:1ml beads) did not produce worse recoveries than ELS cryopreserved at a ratio of 1:4. However, when all CPA media was removed (1:0), viable cell numbers were significantly reduced, whilst per cell function was unaffected. Ideally, the least amount of CPA possible should be used due to the extra thermal mass but not at the expense of recovery. In this study, as per cell function was not affected, it is hypothesised that the decreased viable cell numbers resulted from beads sticking to the inside of the cryovial or pipette as opposed to cell death or reduced proliferation following warming. This could be ameliorated by introducing washing steps into the protocol. Alternatively, it could be argued that this problem is amplified when using small cryovials which are prone to this and that in the final BAL, this "sticking" would be negligible.

The effect of cell density during cryopreservation has previously been investigated on red blood cells. In that study, a greater haematocrit resulted in decreased post-thaw recoveries when slow cooling was applied. The authors suggested that this was due to "cell packing" (i.e. that cells were forced into channels between ice crystals resulting in mechanical disruption) (Pegg et al. 1984). In my study using ELS, the volume of CPA was reduced which may increase injury from cell packing. However, as the HepG2 cell spheroids occupy a relatively small volume within the alginate beads (estimated to be in the region of 10% - unpublished data) the effect of reducing CPA volume is probably less than anticipated for cell suspensions. I would suggest that the perceived loss in cell number using a ratio of 1:0 was due to the methodological issues described above, rather than the effect of cell packing. However, for the purposes of this study, the ELS:CPA ratio selected was 1:1.

As an intermediate step to scale-up, cryopreservation in bags was attempted. Although these bags will hold a far greater volume than the cryovials, and both the width and length of the bags are greater, the depth or thickness of the bag remains similar to that of the cryovial (unlike the culture chamber which is far thicker), which impacts overall heat transfer properties. The thickness of the bag was limited to no greater than 3mm using an aluminium cassette, designed specifically for this purpose (Sputtek et al. 2011). Time-temperature profiles from inside the bag showed that during the initial cooling ramp to -8°C, the vials and bags cooled at similar rates. There was evidence of slight supercooling in both the cryovials and cryobags (a spike in the temperature profile following crystallisation). After nucleation, the cryobag cooled at a slower rate than the cryovial, particularly at the centre of the bag. This could be expected as it is harder to remove heat from a sample of larger volume. Warming profiles of the cryovial and cryobag were very similar and rapid thawing was achieved within both vessels.

Consequently, recovery of ELS was almost identical regardless of whether cryopreserved in cryovials or cryobags. This is an extremely useful finding and demonstrates that cryopreservation within large volumes is possible so long as the dimensions of the freezing vessel of choice are suitable (i.e. that at least one dimension, such as depth in this study, does not exceed ~3mm). Conceivably, cryopreservation could be performed in vessels with an extremely large volume, so long as they were shallow in relatively thin films. Stirling cryocooler technology (discussed in the following Chapter) lends itself to an application such as this where the cooling plates/chamber tend to be small in volume anyway whereas cryogen coolers require large cooling chambers to ensure sufficient cryogen circulation and heat transfer is not particularly efficient (as it occurs via convection as opposed to conduction). Although this could be considered inconsequential for ELS where slow cooling rates are preferable, if more rapid cooling rates were required necessitating better heat transfer, a cryogen-free cooler would certainly be advantageous.

However, in order to deliver a BAL off-the-shelf, cryopreservation and thawing within the culture chamber would be ideal. From looking at the time-temperature profiles of the currently-used chamber during cooling and warming, it is clear that it differs greatly from that of the cryovials, which is not surprising. Firstly, there was no evidence of supercooling; however, a "flattening" in the temperature profile was seen, presumably representing ice formation. It is probable that the characteristic spike was not seen

simply due to the increased mass of the sample masking such a subtle temperature change. During cooling, an average cooling rate of 0.6°C/minute was observed, slower than that of the cryovials. However, from Figures 5-6 and 5-7, it is clear that cell-dense ELS can tolerate slower cooling rates (i.e. 0.3°C/minute) and so this may not be detrimental to ELS recovery following cryopreservation. However, the warming rate achieved in the chamber was far slower than that achieved in either the cryovial or the cryobag and the culture chamber took nearly 2 hours to thaw. Previous data (Figures 3-27, 3-28 and 3-29), shows that such a slow warming rate is far from optimal for ELS.

Indeed, when recovery of ELS cryopreserved in the culture chamber was compared to recovery of ELS cryopreserved in either bags or cryovials, a much poorer recovery was observed. Viability of all cryopreserved ELS was similar at 24 hours post-warming but was lowest in those cryopreserved in the chamber (significantly so when compared to ELS cryopreserved in cryobags). Thereafter, at 48 hours post-warming, viability of ELS cryopreserved in the cryovials and cryobags had improved in agreement with previous findings, earlier in this thesis. To reiterate the hypotheses described in Chapter 3: membrane-intact cells will continue to proliferate to increase FDA staining, whilst nuclei of membrane-damaged cells will degenerate to reduce PI staining, or membrane repair may take place, thereby reducing PI staining. In contrast, those ELS cryopreserved in the culture chamber continued to lose viability, to the degree that almost no cells stained positive for FDA by 48h, indicating that the current protocol was not successful and certainly insufficient for use in a BAL.

As described earlier, it seems more likely that this was due to the failure to achieve a rapid warming rate and that ice recrystallised during warming, lethally damaging ELS. Therefore, an approach to achieve a rapid warming rate was devised. Principally, in addition to warming from the outside the cryopreserved mass, warming from the inside of the mass was also attempted: a warmed liquid was pumped through a coil within the cryopreserved mass. When designing this system, a number of considerations were made.

Firstly, the amount of energy required to melt the cryopreserved mass was calculated. Melting a mass of ice can be split into two stages: firstly, the ice must be warmed to the melting temperature; secondly, the ice must melt (the phase change is endothermic: both of these require heat energy).

The energy required to heat the ice to melting point is a function of the mass of the ice and the initial temperature of the ice (for nitrogen storage -170˚C):

$$E_1 = m \times C_p \times dT = 0.5 \times 1.54 \times 166 = 136.3kJ$$

Where $E_1$ = energy required to heat the ice to melting temperature (kJ)

m = mass (kg) (500g frozen mass)

$C_p$ = heat capacity (kJ/kgK) (average over temperature range)

dT = temperature change (K) (from -170˚C to -4°C)

The energy required to melt the ice is a function of the mass of the ice and the enthalpy of fusion:

$$E_2 = m \times H_f = 0.5 \times 335 = 167.5kJ$$

Where $E_2$ = energy required to melt the ice (J)

m = mass (kg)

$H_f$ = enthalpy of fusion (J/kg)

Therefore, the total energy required to warm and melt the ice is the sum of the two equations above:

$$E = E_1 + E_2 = 136.3 + 167.5 = 303.8kJ$$

The coil was required to transfer this amount of energy within a reasonable time. The rate of heat energy transfer depends on the temperature difference between the tubing and the ice, and the thermal conductivity of tubing, warming liquid and the ice. Therefore, to thaw the culture chamber in a timely fashion (i.e. rapidly), the warming liquid should ideally have:

a) a high temperature (to achieve a high temperature difference between the liquid and the frozen mass)
b) a high specific heat capacity (to minimise the required flow rate)
c) a high mass flow rate (to minimise the change in temperature of the warming fluid to maintain point a) above)
d) low viscosity (to achieve the desired mass flow rate)

However, in reality, there are a number of practical limitations, for example, the warming liquid should:

a) have a low cytotoxicity in case of accidental leakage
b) not be heated above a temperature which could lethally damage cells
c) be liquid within a desired range of temperatures (so that it does not freeze immediately upon contact with the tubing or evaporate at the high temperature)

For example, using water would satisfy the majority of these criteria and it would be practical to use it within a laboratory setting. However, it does not satisfy the last criterion (i.e. would likely freeze upon contact with the frozen tubing). To overcome this, a 50% v/v ethylene glycol solution was used as it satisfies the majority of the criteria (although it has a lower heat capacity than water) and has a lower equilibrium melting point than pure water (-37°C). The fluid temperature of 60°C was chosen as it could be achieved using the waterbaths available, would increase rate of heat transfer without (hopefully) exposing ELS to very high temperatures. From Figure 5-16, it is clear that ELS were not exposed to damaging temperatures and so perhaps a higher temperature of the warming liquid could have been selected that would allow for an increased warming rate.

The tubing must also meet a number of criteria. It should be/have:

- large surface area: so that heat can be transferred to the cryopreserved mass rapidly
- made of heat-conductive material: so that heat can be transferred to the cryopreserved mass efficiently
- low resistance to liquid flow: so that the desired flow rates can be achieve
- biologically inert: so that it does not damage the biomass in any way

For the purposes of this thesis, copper met these criteria sufficiently and so was used in this study. It is also readily machined which was advantageous here. In the final BAL, it would be convenient if the tubing could be disposable (along with the rest of the BAL) and so a plastic would perhaps be preferable. However, plastics tend to have poor thermal conduction properties and there may be issues regarding leaching upon contact with either DMSO or ethylene glycol (if used).

The design of the shape of the tubing itself was also important. The design here was chosen as it was relatively simple to machine, divided the cryopreserved mass into smaller portions, and was made up of relatively short lengths of tubing. The use of

short lengths of tubing is useful here as it reduces resistance to flow ensuring that a high flow rate could be achieved.

The required flow rate depends on the temperature change of the fluid as it passes through the coil. This ultimately depends on the coil design and would need to be determined experimentally. However, to demonstrate the relationship between temperature change of the warming fluid and flow rate, Figure 5-20 has been plotted using the equation below:

$$dQ/dt = dm/dt \times C_p \times dT$$

Where $dQ/dt$ = heat transfer rate (J/s) ($dQ$ = 303.8kJ)

$dm/dt$ = mass transfer rate (kg/s)

$C_p$ = heat capacity (J/kgK) (3.265kJ/kgK)

$dT$ = temperature change (K)

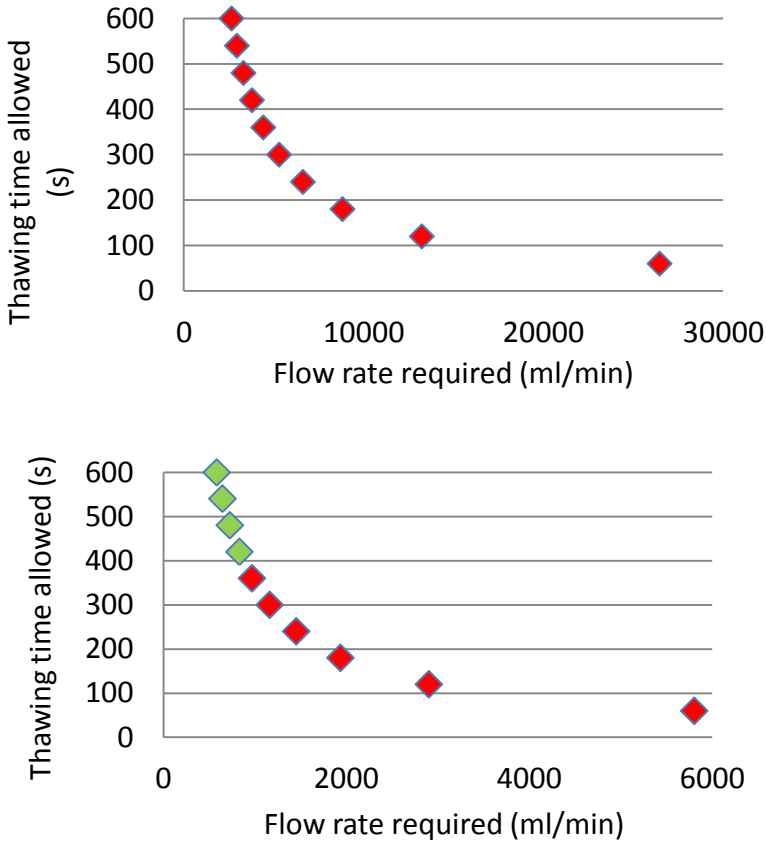


**Figure 5-20. Relationship between flow rate and thawing time for culture chamber.**

**Mathematical modelling of culture chamber thaw if warming liquid is allowed to change temperature by only 4K (top) or 32K (bottom). Red diamonds indicate impractical flow rates whereas green diamonds indicate achievable flow rates.**

As the graphs show, if the temperature of the warming liquid only changes by 4K, the flow rate required to achieve thawing within 10 minutes is impractical. Whereas, if the temperature of the warming liquid drops by 32K, the flow rate required to achieve thawing within 10 minutes becomes feasible (Figure 5-20).

The flow rate can be changed as needed using an external pump system, however this is limited by the speed at which the pump can work (above 400ml/min was impractical using the pump available). For testing of the prototype coil, a flow rate of 400ml/minute was selected as this should allow rapid thawing to be achieved (subject to experimental testing of the coil design).

The system was tested for thawing a cryopreserved mass in the culture chamber, without ELS. In this dummy run, thawing was achieved within 10 minutes, far quicker than the 2 hours taken without the modified warming system. Although 10 minutes is longer than the 2 minutes taken for cryovial and cryobag thawing, this is certainly an improvement and previous results (Figures 3-27, 3-28 and 3-29) indicate that the recovery of ELS thawed at this rate may be sufficient for a BAL. This thawing rate would be further improved if the temperature of the warming liquid could be maintained at 60°C (which did not happen in this study as the waterbath was unable to do so) and if the coil design were further optimised. It should of course be noted, that a larger volume of cryopreserved biomass (i.e. 1-2kg) would require thawing for the final BAL but this demonstrates that the strategy is valid. However, when this system was tested on ELS, the system failed technically. This was likely due to stress/strain impact on the coil arising from the initial cryogenic experiment which, when repeated, caused failure in the materials. This highlights the need for a more robust design and unfortunately the efficacy of this new design could not be assessed within the timeframe of this thesis.

Clearly, the scale up of the cryopreservation protocol requires further work and this is an area on which future work would focus.

## 5.6. Conclusions

Successful cryopreservation of cell-dense ELS is more difficult to achieve than less cell-dense ELS. This is probably due to the increased number of cells contained within individual spheroids which makes the dehydration process (to decrease possibility of IIF) both longer and more challenging.

Cryopreservation of Inotech-encapsulated ELS was easier to achieve than JetCutter-encapsulated ELS. This may be due to differing cell-cell contact or spheroid shape which makes achieving sufficient dehydration more complex.

Transfer of the cryopreservation protocol from cryovials to cryobags is achievable as temperature control can be maintained accurately and evenly, despite the increased cell mass. This is true both during cooling and warming.

Transfer of the cryopreservation protocol into a culture chamber as would be ideal for the final BAL is more complex, with temperature control during both cooling and warming being more difficult to achieve. It seems that the failure to thaw ELS rapidly from cryogenic temperatures is more problematic than the inability to cool ELS at the optimal rate.

Thawing of the culture chamber can be achieved within a suitable timeframe using a modified warming system. This is an area for future work.

# CHAPTER 6

# Regulatory Considerations

## 6.1. Introduction

The overall aim of this thesis was to develop a method for cryopreservation of hepatocytes to comprise the cellular component of a bioartificial liver (BAL). As the BAL would eventually be used to treat human patients suffering from acute liver failure (ALF), a number of healthcare regulations must be adhered to. These are stipulated by governing bodies such as the Medicines and Healthcare products Regulatory Agency in the UK, the Food and Drugs Administration in the US and the Japanese Compliance and Medical Approval Agency in Japan. The exact requirements of these agencies differ (and many fall beyond the scope of this thesis, such as cell source and culture matrix) but some were considered in this Chapter, specifically those listed in the following section.

### 6.1.1. Use of fresh frozen plasma in place of foetal calf serum during cryopreservation

Foetal calf serum (FCS) has long been used for successful cryopreservation of cells from various tissues and species. The exact mechanisms by which FCS acts to protect cells during cryopreservation remain unknown but it is accepted that plasma membranes are better maintained during the cryopreservation process in the presence of FCS. FCS has been shown to be beneficial specifically for cryopreservation of liver cells in numerous culture formats including: suspensions of duck embryo hepatocytes (Schacke et al. 2009); monolayer cultures of adult human hepatocytes (Stevenson et al. 2004); and even slices of adult human liver (Fisher et al. 1993).

Unfortunately, the use of xenogeneous components during cryopreservation of cells or tissues eventually intended for treatment of humans is undesirable due to concerns regarding infection and immune reaction. Human serum has been suggested as an allogeneic alternative to FCS and has been used during cryopreservation of various cells and tissues, with comparable success, notably in ovarian tissue (Fabbri et al. 2006). Similarly, as one of the suggested protective mechanisms of serum is membrane stabilisation by plasma proteins, the presence of human serum albumin alone (as

opposed to serum) has been demonstrated to offer similar protection to serum (Hreinsson et al. 2003).

No information regarding the use of FFP as an FCS alternative during cryopreservation of any cell type could be found. ELS are cultured using FFP in place of FCS within the bioreactor setup, and FFP has been previously demonstrated to increase both proliferation and function of ELS (Erro et al. 2010) compared to FCS culture. The exact components of FFP that result in improved cell culture are unknown but it is likely to result from a combination of macromolecules. FFP is also already used to treat patients suffering from ALF (plasmapheresis to remove toxins). Although it has not been previously demonstrated, it is likely, and suitable in this instance, that FFP may convey protection to ELS during cryopreservation and this was investigated.

### 6.1.2. Use of DMSO during cryopreservation

Cryopreservation of ELS will undoubtedly require the use of a cryoprotectant (CPA) to protect against the damaging effects of cryopreservation. DMSO is routinely used for cryopreservation of many cell and tissue types including hepatocytes (Diener et al. 1993; Terry et al. 2010). Indeed, throughout this thesis, good functional recoveries have been demonstrated using 12% DMSO as cryoprotectant. DMSO is relatively small and therefore readily able to cross the cell membrane to decrease the likelihood of intracellular ice formation within ELS. Experimentally, when DMSO was compared to other commonly-used CPA, it has shown to be more rapidly taken up by various cell types and tissues including sperm (Cooper et al. 2008) and cartilage (Jomha et al. 2009). Similarly, it has also been demonstrated to increase hydraulic conductivity better than other CPA in oocytes (Karlsson et al. 2009). Both of these properties may convey even more protection here in multicellular ELS. Rapid permeation throughout entire individual spheroids is necessary to avoid intracellular ice formation and potential subsequent propagation. Similarly, by increasing hydraulic conductivity, dehydration may occur more rapidly, again reducing likelihood of IIF.

Despite the routine use of DMSO, it is known to be cytotoxic with increasing concentration, exposure time and temperature (Hak et al. 1973; Wang et al. 2007). This in itself means that there is a possibility that DMSO may injure ELS prior to cryopreservation (upon addition) and if not removed from ELS post-cryopreservation may cause reduced recovery of ELS in post-thaw cultures or pose a risk to the BAL recipient. Similarly, this risk has also been considered for cryopreservation of

hepatocytes intended for transplantation where DMSO is also used as CPA. As a result, it is recommended that hepatocytes are cryopreserved at high cell densities such that the volume of DMSO transplanted (along with cells) into the recipient is minimised (Hengstler et al. 2000; Terry et al. 2010).

For these reasons, it would be desirable to reduce the concentration utilised for cryopreservation of ELS. It is recognised that concentrations at or above 12% DMSO have been previously shown to be optimal for cryopreservation of hepatocytes (Terry et al. 2010) but hypothesised that these reports result from incomplete DMSO permeation at lower concentrations. It may, therefore, be possible to reduce DMSO concentration by increasing CPA incubation time prior to cryopreservation. An additional strategy may be to include non-permeating CPA, such as raffinose or sucrose, to further promote ELS dehydration, in the hope that this may counterbalance the reduced DMSO concentration. Both of these approaches were investigated here.

### 6.1.3. Use of GMP-compliant preservation media during cryopreservation

There are numerous preservation media available but they can broadly be classed into 2 groups: those intended for storage of whole organs or tissues at temperatures in the region of 4-25°C; and those intended for long-term storage of cells or tissues at very low temperatures (i.e. in liquid nitrogen or in vapour phase of liquid nitrogen). Organ preservation media are osmotically-balanced, pH appropriate solutions. They may also contain antioxidants to counteract oxidative stress associated with cold storage and ischaemia.

One of the most commonly used, and considered to be the gold standard for liver and kidney preservation, is University of Wisconsin (UW) solution (Feng et al. 2007). UW solution contains a high concentration of potassium (120mM), low sodium (30mM), hydroxyethylstarch (5% w/v) and glutathione (Muhlbacher et al. 1999). The presence of starch, included to prevent oedema, is sometimes criticised as it results in increased viscosity which may decrease perfusability, and here, may reduce miscibility with cryoprotectants. Similarly, the sodium/potassium concentrations are reversed compared to that expected under physiological conditions and this has been associated with endothelial cell damage. Kidney Perfusion Solution (KPS) differs in that it does not contain starch, has the "correct" concentrations of sodium and potassium but does contain antioxidant glutathione.

Cryopreservation media, such as CryoStor®, Profreeze® and Synth-a-Freeze® have also been developed in recent years. These solutions are designed specifically for use during cryopreservation and long-term storage at cryogenic temperatures. As a result some already contain 10% DMSO as cryoprotectant. The compositions of these media are unknown due to Intellectual Property rights but contain or recommend addition of DMSO prior to use.

### 6.1.4. Use of liquid nitrogen during cryopreservation

Avoiding the use of liquid nitrogen during cooling to and storage at cryogenic temperatures is desirable for a number of reasons. These include safety (personnel must be trained in its use and areas must be well-ventilated) but also cross-contamination is a potential hazard (viral transmission has been observed between samples stored in liquid nitrogen) resulting in the recommendation that samples be stored in the vapour phase of liquid nitrogen where contamination risks are reduced. Furthermore, recently introduced EU regulations state that liquid nitrogen used for cryopreservation of cell-therapy products, such as a BAL, should be of pharmaceutical grade ("The Rules Governing Medicinal Products in the EU", 2010), which commercially-available liquid nitrogen is not.

#### 6.1.4.1. Avoiding the use of nitrogen during cooling

Liquid nitrogen is traditionally used during cooling as it allows good, reliable and reproducible control of sample cooling via a solenoid valve in a feedback loop. This temperature control is vital for successful cryopreservation where cells are sensitive to applied cooling rate, such as ELS. However, an alternative cryogen-free approach to cryopreservation has been developed: a Stirling cryocooler based upon Stirling engine technology.

Stirling engines work by cyclic compression and expansion of a gas at different temperatures, converting heat energy to work. There are various setups that can be used to achieve this and one is shown in Figure 6-1.

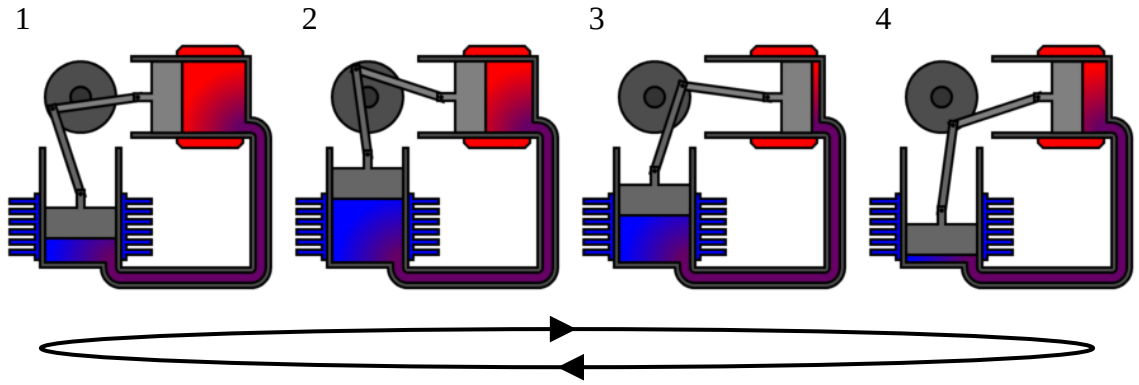


**Figure 6-1. Stirling engine operation.**

**Heat is applied to the "hot" cylinder causing expansion of gas in this cylinder (1). This gas expands into the "cold" cylinder which forces the piston up (2) causing compression of gas in the "hot" cylinder (3) which is heated and expands as a result (4). The cycle continues, producing more work energy.**

However, this cycle can work in the opposite direction. By putting in work energy, using a motor, the cycle can progress without application of heat, resulting in repeated expansion and compression of the working gas as the pistons move. Whilst a compressed gas will give off heat to the surrounding environment, an expanded gas will draw heat in from the environment. Stirling cryocoolers exploit this and use the expanded gas (in the "cold" chamber in Figure 6-1) to remove heat from a cooling plate on which samples to be cryopreserved are postioned.

The EF600 is one such Stirling-engine based cryocooler that could be used for controlled rate freezing for cryopreservation. The EF600 is capable of cooling samples down to -100°C. It has already been demonstrated to be effective for cryopreservation of suspensions of stem cells, sperm cells and even multicellular embryos (Faszer et al. 2006; Morris et al. 2006) but has not been trialled for cryopreservation of hepatocytes, as either single cells or as 3D cultures.

#### 6.1.4.2. Avoiding the use of nitrogen during long-term storage at low temperatures

Whilst cryopreservation of ELS with good recovery of function has been demonstrated possible, in order to supply ELS to the clinic as required, long-term storage temperature must be considered. Whilst long-term storage in either nitrogen or in the vapour phase of liquid nitrogen is considered the gold-standard, these facilities are not always available, there is a risk of contamination, and safety requirements must be considered.

Therefore, avoiding nitrogen use entirely is preferable so it would be facilitative to the production of a BAL if long-term storage at -80°C was sufficient.

Few prospective studies have investigated the effect of long-term storage at -80°C and results appear to be conflicting. Some have reported that -80°C storage is inferior to that of in the vapour phase of liquid nitrogen (Feng et al. 1996; Kashima et al. 1999) for heart valves. However, in a different cell type (peripheral blood mononuclear cells) storage at -80°C was reported to yield acceptable recoveries for up to 18 months (although recovery did decline beyond this storage time) (Valeri & Pivacek 1996).

## 6.2. Aims

The aims of this chapter were to evaluate whether it was possible to cryopreserve ELS with good functional recovery whilst giving consideration to regulations mandated by governing bodies worldwide. The following were considered:

**alternatives to the use of xenogeneic serum:** The use of xenogeneic serum during cryopreservation is common but is not ideal for use in this application. Therefore, serum alternative fresh frozen human plasma (FFP) was investigated.

**reducing the concentration of DMSO:** The use of low concentrations of DMSO during cryopreservation would be preferable in this application to minimise the risk of this potentially toxic chemical being unintentionally returned to the patient.

**the use of good manufacturing practice (GMP) compliant cryopreservation media:** As dictated by the governing bodies, any cryopreservation media should comply to GMP (i.e. should be chemically-defined and free of animal components). A number of GMP-compliant cryopreservation/organ preservation media are commercially available and these were trialled for efficacy.

**avoiding the use of liquid nitrogen during cryopreservation and storage:** Recently introduced regulations state that nitrogen used at any stage of cryopreservation, including during cooling and storage, should be of pharmaceutical grade. An alternative, cryogen-free approach, to cryopreservation was investigated.

## 6.3. Methods

Following cryopreservation, ELS were assessed for recovery using the methods described in Chapter 2 at 24 hours post-warming.

### 6.3.1. Effect of FFP on ELS recovery

ELS were cryopreserved using the Kryo 10 using a non-linear cooling protocol (Diener et al. 1993). Standard CPA media was supplemented with FFP over a range of concentrations as indicated in the Results section of this Chapter. ELS were stored in the vapour phase of liquid nitrogen, thawed rapidly and returned to culture, before recovery was asssessed.

### 6.3.2. Reducing DMSO concentration during cryopreservation

ELS were exposed to DMSO at concentrations of either 5 or 12% alone or 5% DMSO with 300mM raffinose for between 15 minutes (standard) and 1 hour on ice. ELS were cryopreserved using a non-linear cooling protocol (Diener et al. 1993) in the Kryo 10 cooler, thawed rapidly and returned to culture.

### 6.3.3. Use GMP-compliant CPA media

Materials

DMSO
UW solution (Organ Recovery Systems)
KPS (Organ Recovery Systems)
CryoStor (Biolife)
Profreeze (Lonza)
Synth-a-Freeze (Cascade Biologics)
Cholesterol

Method

ELS were cryopreserved in the Kryo 10 CRF using a non-linear cooling protocol (Diener et al. 1993). ELS were cryopreserved in either 12% DMSO v/v with either UW solution, Kidney Perfusion Solution (KPS), CryoStor, Profreeze or Synth-a-Freeze as vehicle solutions with 1.1mg/ml cholesterol as nucleator. CryoStor and Synth-a-Freeze already contained 10% DMSO but this concentration was raised to 12% DMSO. ELS

were stored in the vapour phase of liquid nitrogen, thawed rapidly and returned to culture.

### 6.3.4. Avoiding the use of liquid nitrogen during cooling

6.3.4.1. Can the EF600 perform a multi-step cooling profile?

Method

In order to test if the EF600 could apply a multi-step cooling profile evenly across the entire headplate, Type K thermocouples were taped to the bottom of the wells of the headplate of the EF600, in 4 locations from edge to middle, as shown in Figure 6-2. A non-linear cooling protocol (modified from Diener et al. 1993) was programmed using iTools software (EuroTherm). Temperature of the headplate was logged every 10 seconds using the TC-08 datalogger.

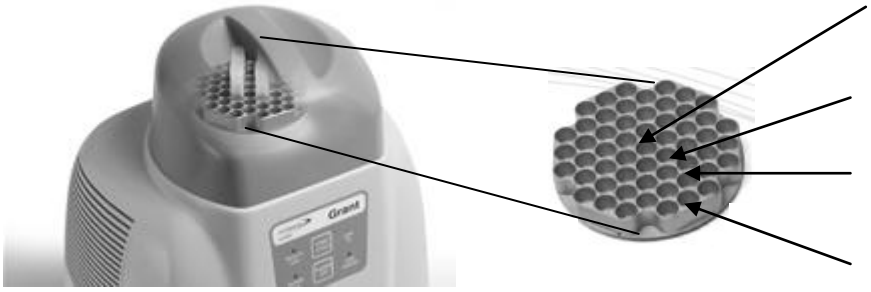

**Figure 6-2. EF600 headplate.**

**Arrows indicate location where Type K thermocouples were attached to the headplate.**

6.3.4.2. Can the EF600 apply a multistep cooling profile to cryovials?

In cryogen-cooled CRFs, nitrogen vapour is circulated throughout the entire CRF chamber and samples are cooled by convection. However, in the EF600, cooling is achieved by conduction of heat away from the sample via the headplate and so the ability of the EF600 to cool vials was tested.

Method

To test efficacy of the conduction method, the EF600 was used to apply a non-linear cooling protocol (modified from Diener et al. 1993) to empty cryovials placed in 4 different locations across the headplate as in Figure 6-2. Temperatures at the base and

top of the cryovials were measured and logged every 10 seconds using the TC-08 datalogger.

6.3.4.3. Does cholesterol function as a heterogeneous nucleator in the EF600?

Method

1.25ml volumes of 12% DMSO/Celsior with 1.1mg/ml cholesterol were cooled using the EF600. Temperatures at the base of the cryovials were measured and logged every 10 seconds using the TC-08 datalogger.

Method

As a result of the experiment above, the method was modified slightly: to test if cholesterol could act as heterogeneous nucleator in smaller samples (i.e. where the temperature gradient was minimised), a repeat of the experiment above was performed differing only in that the sample volume was reduced to 500µl.

6.3.4.4. How does the EF600 compare to a cryogen cooler?

As the EF600 was able to perform reliably and reproducibly using small volumes, it was compared directly to the nitrogen-cooled Kryo 10 CRF.

Method

ELS were cultured for 8 days before being cryopreserved using identical cryoprotectants (CPA) (12% v/v DMSO in Celsior solution with 1.1mg/ml cholesterol) and cooling profiles (non-linear cooling protocol (modifield from Diener et al. 1993)). As it was recognised that the EF600 was not capable of cooling 1.25ml volumes of sample, 500µl samples were used in this study. ELS (from both cohorts) were stored in the vapour phase of liquid nitrogen for 1 week before thawing and return to culture. ELS were cultured for 72 hours post-warming and viability, cell number and function compared to an unfrozen control at equivalent time points.

### 6.3.5. Can ELS be stored without nitrogen for extended time periods?

Method

ELS were cultured for 8 days before being split into 2 groups and cryopreserved in 12% DMSO in UW using the Kryo 10 CRF. Both groups were cryopreserved using a non-linear cooling protocol (Diener et al. 1993). However, the first group was removed from the CRF once the chamber had reached -80°C and transferred to the back of a -80°C freezer and stored within in a cotton wool-lined polystyrene box. The second

group was removed from the CRF once the profile was complete (ie. the chamber had cooled to -160°C) and transferred to the vapour phase of liquid nitrogen. Both groups were stored for up to 1 year and vials removed after 1, 2, 3, 6, 9 and 12 months. ELS were thawed, returned to culture and assayed for viability, cell number and function after 24 hours.

6.3.5.1. Differential scanning calorimetry

To determine the glass transition temperature (Tg) of the CPA mixture used for these studies, mDSC was utilised. The general method for this technique is more fully described in Chapter 2, section 2.10. Briefly, approximately 80µl aliquots of the CPA mix were analysed at 5°C/min with modulation of 1°C/min.

## 6.4. Results

### 6.4.1. Effect of fresh frozen plasma on cell viability, number and function in post-warming cultures

ELS cryopreserved in 0% FFP were approximately 30% viable 24h post-warming. A significant improvement in ELS viability was achieved using 10% FFP supplementation. At concentrations greater than this, viability was not significantly different to 0% FFP, with the exception of 50% FFP supplementation, where a small improvement was observed (Figure 6-3).

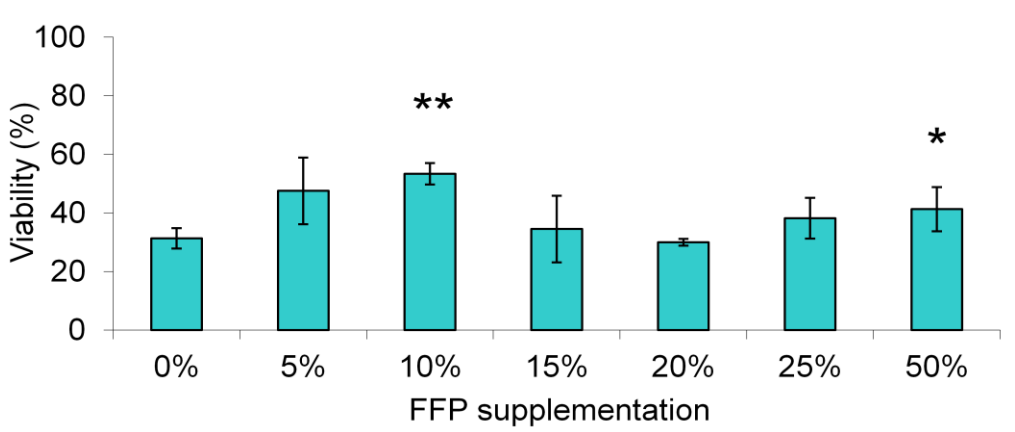


**Figure 6-3. Effect of FFP on ELS viability in post-warming cultures.**

**ELS were cryopreserved in 12% DMSO/UW with FFP supplementation at 0, 5, 10, 15, 20, 25 or 50% using a multi-step cooling profile (Diener et al. 1993). ELS were warmed rapidly using a 37°C waterbath and viability measured 24 hours post-warming. Data are means of n=5 +/- SD from a single experiment. * $p<0.05$, ** $p<0.01$, when compared to 0% FFP.**

Viable cell numbers and total function data displayed similar trends with 10% FFP supplementation being optimal but at concentrations greater than this, cell numbers were significantly decreased cf. 10% Figures 6-4 and 6-5.

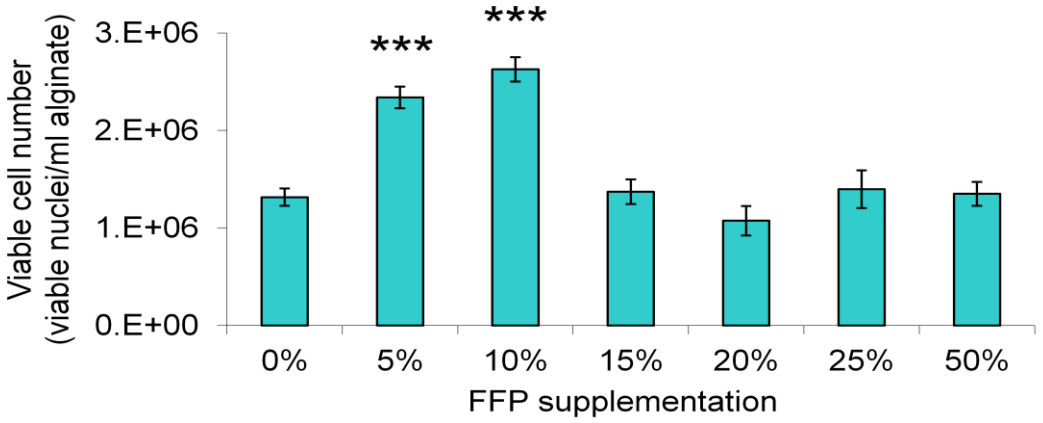


**Figure 6-4. Effect of FFP on ELS viable cell numbers in post-warming cultures.**

**ELS were cryopreserved in 12% DMSO/UW with FFP supplementation at 0, 5, 10, 15, 20, 25 or 50% using a multi-step cooling profile (Diener et al. 1993). ELS were warmed rapidly using a 37°C waterbath and viable cell numbers were measured 24 hours post-warming. Data are means of n=5 +/- SD from a single experiment. *** p<0.005 when compared to 0% FFP.**

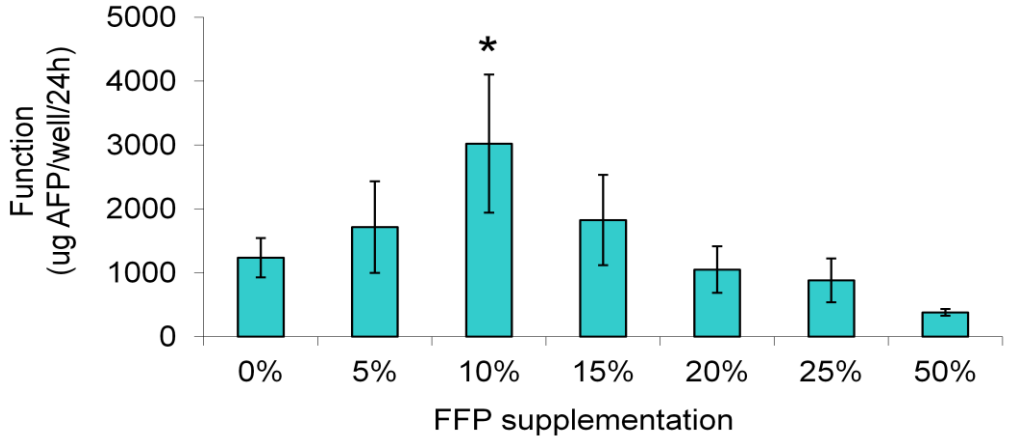


**Figure 6-5. Effect of FFP on ELS synthetic function in post-warming cultures.**

**ELS were cryopreserved in 12% DMSO/UW with FFP supplementation at 0, 5, 10, 15, 20, 25 or 50% using a multi-step cooling profile (Diener et al. 1993). ELS were warmed rapidly using a 37°C waterbath and function was measured 24 hours post-warming. Data are means of n=5 +/- SD from a single experiment. *p<0.05 when compared to 0% FFP.**

### 6.4.2. Effect of reducing DMSO during cryopreservation

Optimal viability was achieved using the standard cryopreservation protocol (i.e. 15 minutes in 12% DMSO on ice). Viability in ELS exposed to 12% DMSO dropped with

extended exposure time to a minimum after 1 hour to approximately two thirds that of 15 minute exposure. Viability using 5% DMSO was lower (with or without raffinose) cf. 12% DMSO although no correlation between exposure time and viability was observed. Viable cell numbers were also optimal using the standard cryopreservation protocol. Again viable cell numbers decreased with exposure time to 12% DMSO. ELS pre-incubated with 5% DMSO (either with or without raffinose) showed increased viable cell numbers to a maximum after 1 hour pre-incubation. The presence of raffinose did not affect viable cell numbers. Functional data also reflected this but again optimal function was achieved using the standard cryopreservation protocol (Figure 6-6).

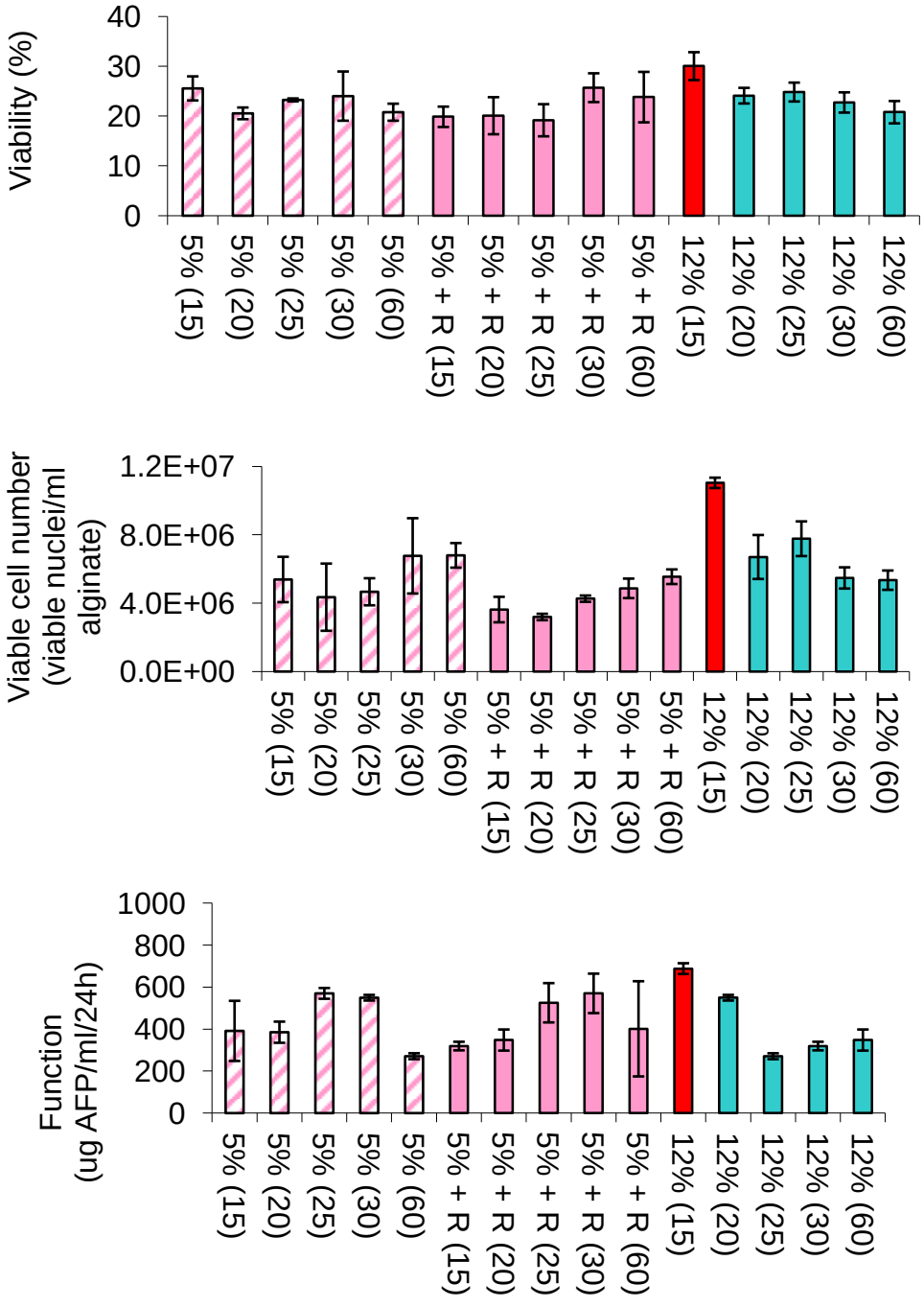


**Figure 6-6. Effect of reduced DMSO concentration and raffinose on ELS recovery in post-warming cultures.**

**ELS were incubated prior to cryopreservation in either 5 or 12% DMSO as indicated. +R indicates addition of 300mM raffinose prior to cryopreservation. (x) indicates minutes that ELS were exposed to DMSO. Red bars indicate the current cryopreservation protocol. All incubation steps were performed on ice. ELS were cooled using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Cell viability (top), viable cell number (middle) and function (bottom) were measured 24 hours post-warming. Data is expressed as mean n=3 +/- range from a single experiment.**

### 6.4.3. Use of different GMP-compliant cryopreservation media

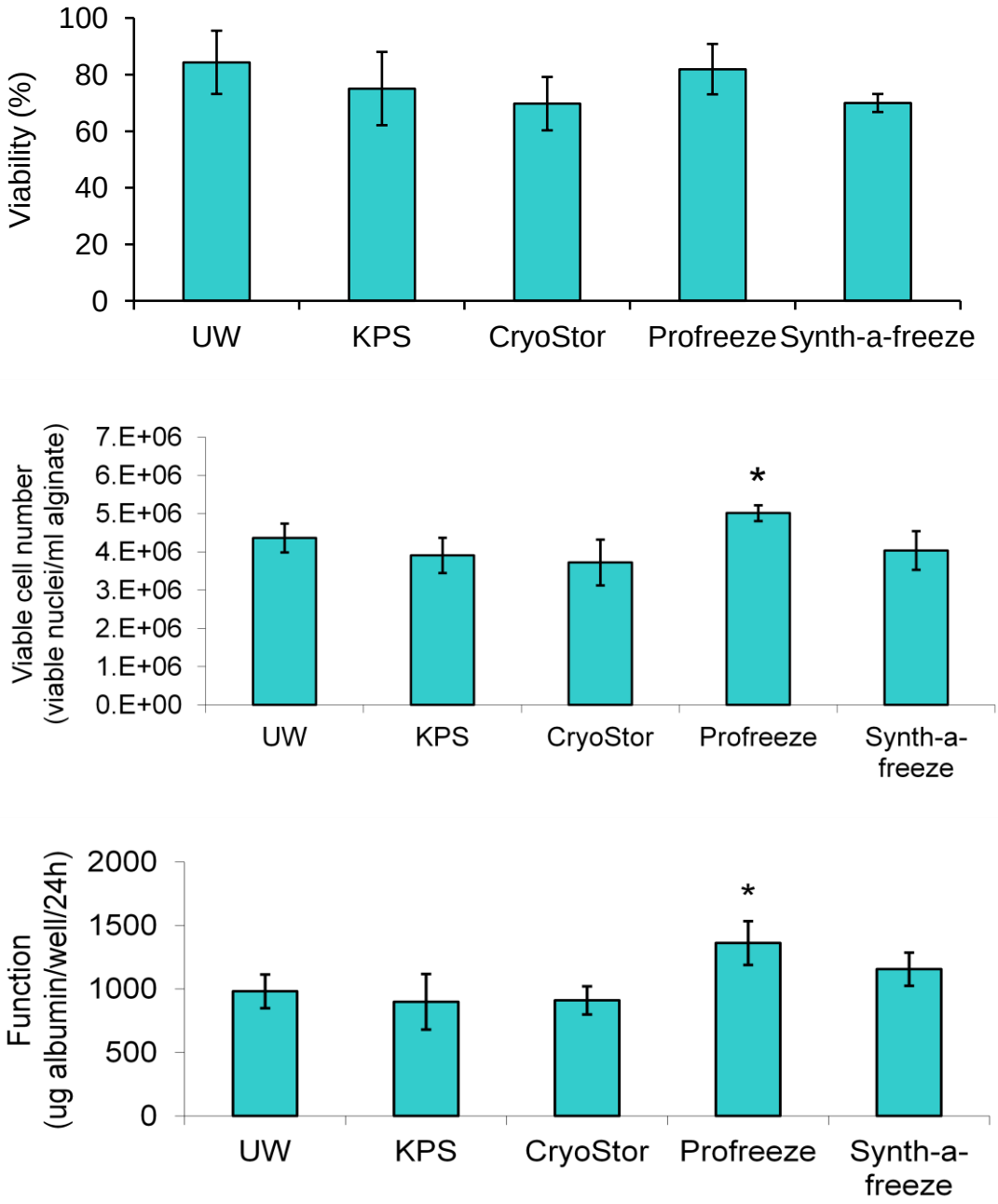


**Figure 6-7. Effect of different cryopreservation media on ELS recovery in post-warming cultures**

**ELS were cryopreserved in 12% DMSO in 5 different preservation media with cholesterol using a multi-step cooling profile (Diener et al. 1993). ELS were warmed rapidly (37°C waterbath) and cell viability (top), viable cell number (middle) and function (bottom) were measured 24 hours post-warming. Data is expressed as mean for n=5 +/- SD from a single experiment. * $p<0.05$ compared to other groups.**

Viability was unaffected by preservation media used but was high in all groups at approximately 75% on average. Cell numbers and viable cell numbers were similar between all groups with the exception of ELS cryopreserved in Profreeze where a small but significant improvement in viable cell number was observed. Functional data also showed a significantly increased albumin production (~1.5 fold) in ELS cryopreserved in Profreeze cf. the other groups (Figure 6-7 on the previous page).

### 6.4.4. Use of EF600 during cooling

6.4.4.1. Can the EF600 perform a multi-step cooling profile?

Cooling was applied uniformly across the entire headplate. However, the EF600 was not able to perform the "shock cooling" ramp of the multistep cooling profile and a small overshoot and modulation was observed following this ramp (Figure 6-8 below).

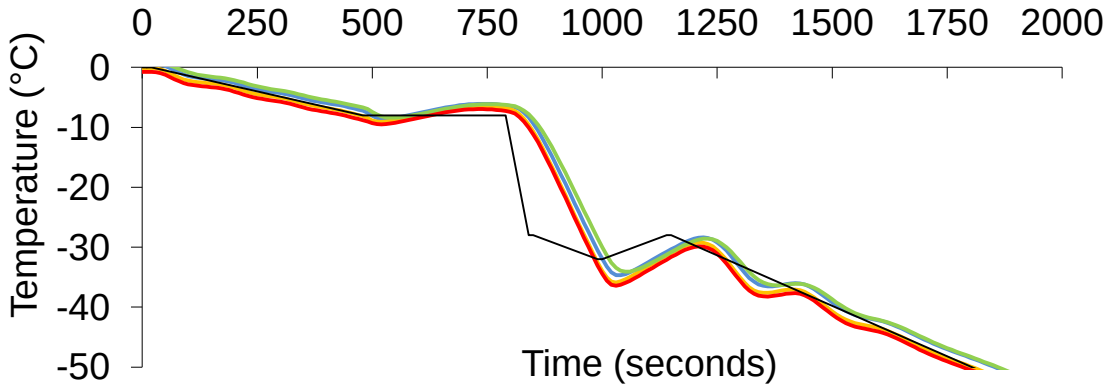


**Figure 6-8. Temperature of the EF600 headplate at different locations during a multistep cooling profile.**

**The 4 different locations were middle plate (red), near middle (blue), near edge (orange) and edge (green). The black line indicates programmed cooling profile (modified from Diener et al. 1993 to include a 5 minute hold at -8°C to ensure samples had reached -8°C before onset of the shock-cooling ramp. This graph is a typical representation of 4 identical experiments performed.**

6.4.4.2. Can the EF600 apply cooling to cryovials?

The temperature profile showed that the EF600 was able to effectively conduct heat away from the lower portion of the cryovial, enabling application of the desired cooling profile. However, temperatures measured at the top of the cryovial revealed that cooling was not achieved as desired (see Figure 6-9).

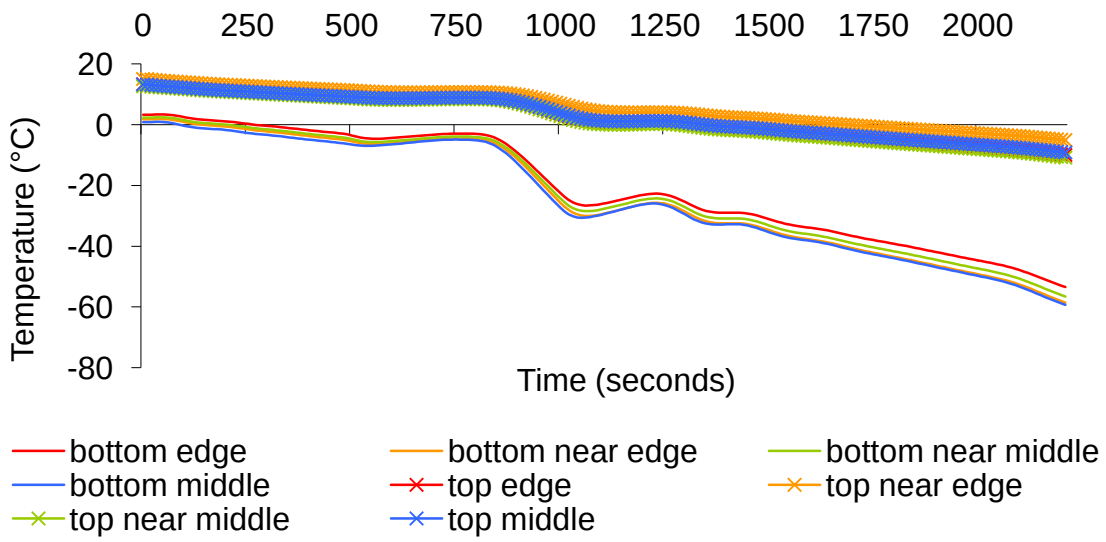


**Figure 6-9. Temperature variation within empty cryovials cooled using the EF600 during application of a multistep cooling profile.**

**These profiles are taken from a single experiment.**

6.4.4.3. Does cholesterol maintain efficacy in the EF600?

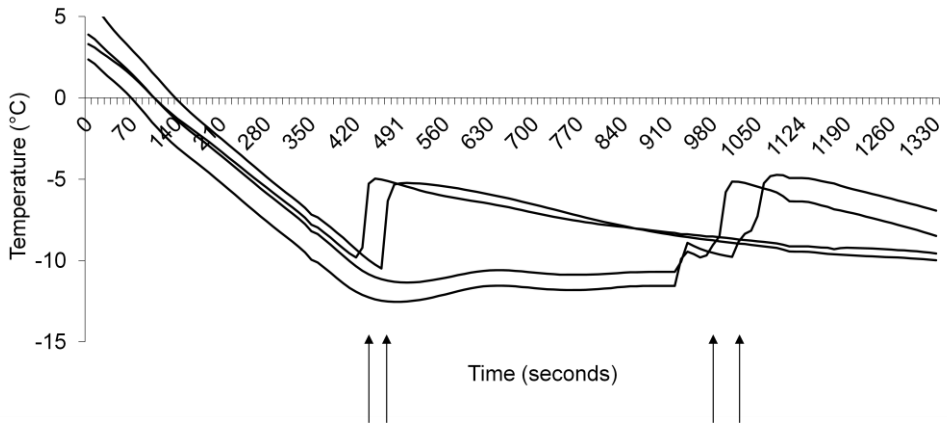


**Figure 6-10. Effect of cholesterol on nucleation temperature in the EF600 in 1.25ml volumes.**

**Temperature profiles of 4 separate 1.25ml samples contained in cryovials cooled using the EF600 during application of a multistep cooling profile (modified from Diener et al. 1993) within a single experiment. Arrows indicate 4 occasions when cryovials were inverted.**

Supercooling of approximately 5°C was observed, greater than that observed within the cryogen cooler (Figure 5-10). However, by inverting the cryovial (at times indicated by arrows), nucleation could be triggered (Figure 6-10 above).

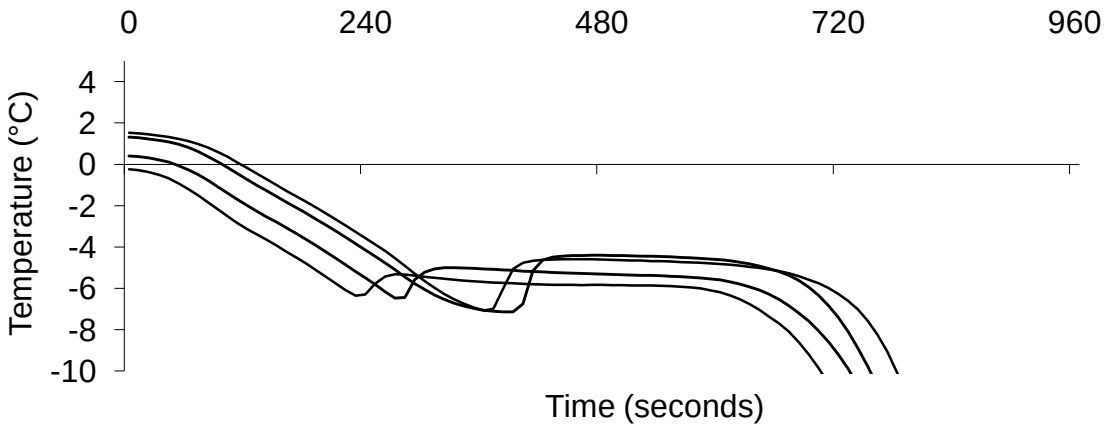


**Figure 6-11. Effect of cholesterol on nucleation temperature in the EF600 in 500µl volumes.**

**Temperature profiles of 4 separate 500µl samples contained in cryovials cooled using the EF600 during application of a multistep cooling profile (modified from Diener et al. 1993).**

When smaller samples (i.e. 500µl) were used, supercooling was reduced to 1-2°C, in line with previous data using the cryogen-cooler (Figure 5-10). Figure 6-11 above shows the time-temperature profile for this experiment.

6.4.4.4. How does the EF600 compare to a cryogen cooler?

Results for both cryopreserved cohorts are expressed as fold change compared to the unfrozen control.

Viability of recovered ELS was high in both cryopreserved groups at all time points at approximately 0.9-fold or greater that of the unfrozen control. Viable cell numbers of both cryopreserved groups were approximately 0.7-fold that of the control. ELS viable cell numbers cryopreserved using the Kryo10 were slightly higher than those cryopreserved using the EF600 at all time points, although this was only significant at 48h post-warming. Function, as assessed by total albumin production, was approximately half that of the unfrozen control over the 72 hour period but there was no significant difference between the 2 cryopreserved groups. These results are shown below and in Figure 6-12.

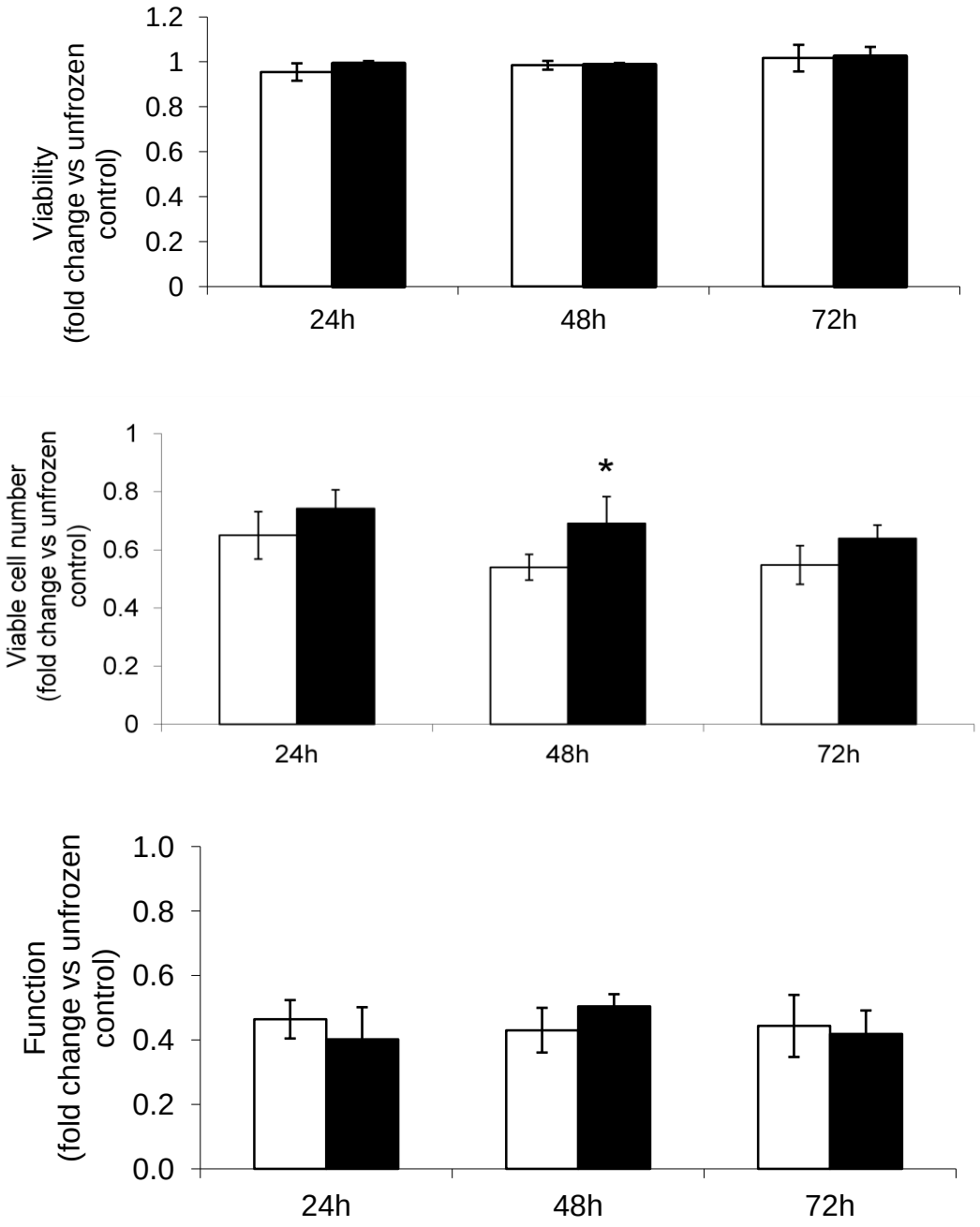


**Figure 6-12. Comparison of ELS recovery in post-warming cultures following cryopreservation in either Kryo10 or EF600.**

**ELS were cryopreserved in 12% DMSO/Celsior with cholesterol in either the EF600 (white bars) or Kryo 10 (black bars) using a multi-step cooling profile modified from Diener et al. 1993) in 500µl volumes. Cell viability (top), viable cell number (middle) and function (bottom) were measured at 24, 48 and 72 hours post-warming. Results are expressed as compared to unfrozen control at equivalent time point. Data are means n=5 +/- SD from 5 independent experiments. *p<0.05, EF600 compared to Kryo 10 at the same time point. Taken from Massie et al. 2011b.**

### 6.4.5. Comparison of storage at -80°C and in the vapour phase of liquid nitrogen

Viability of both cryopreserved groups decreased between 1 and 12 months storage, although this was only statistically significant for ELS stored at -80°C, where viability fell over time to approximately 50% out to 1 year. Furthermore, there was no trend to decreased viability with increased storage time for ELS stored in the vapour phase of liquid nitrogen. This was characterised by increased levels of PI staining relative to FDA staining and evidence of FDA leakage from membrane-damaged ELS. Viability of ELS was significantly improved by storage at -150°C cf. -80°C at all time points.

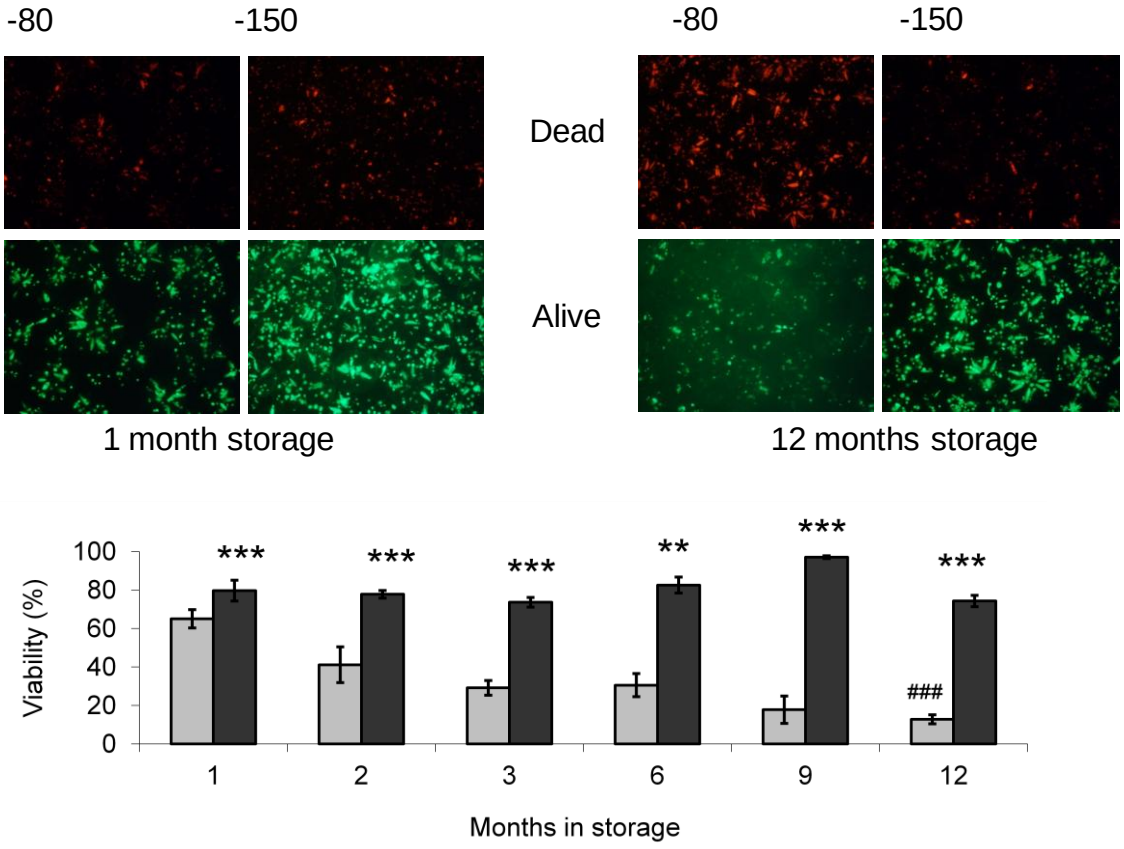


**Figure 6-13. Effect of storage temperature on ELS viability in post-warming cultures.**

**ELS were cryopreserved in 12% DMSO/UW with cholesterol using a multi-step cooling profile (Diener et al. 1993) and stored either in the vapour phase of liquid nitrogen (-150°C) (dark grey bars) or at -80°C light grey bars). ELS were warmed rapidly using a 37°C waterbath. Viability was assessed 24h post-warming both qualitatively (top) and quantitatively (bottom). Micrographs show FDA (live) and PI staining after storage time indicated. x4 original magnification. **p<0.01, ***p<0.005 -150°C compared to -80°C at same time point. ###p<0.005 compared to 1 month storage.**

Viable cell numbers were maintained for the entire 12 months in ELS stored at -150°C. Conversely, viable cell numbers of ELS stored at -80°C showed a progressive decline in number to a minimum after 12 months by which time only ~20% viable cell population remained. At all time points, viable cell numbers were significantly improved by storage at -150°C cf. -80°C.

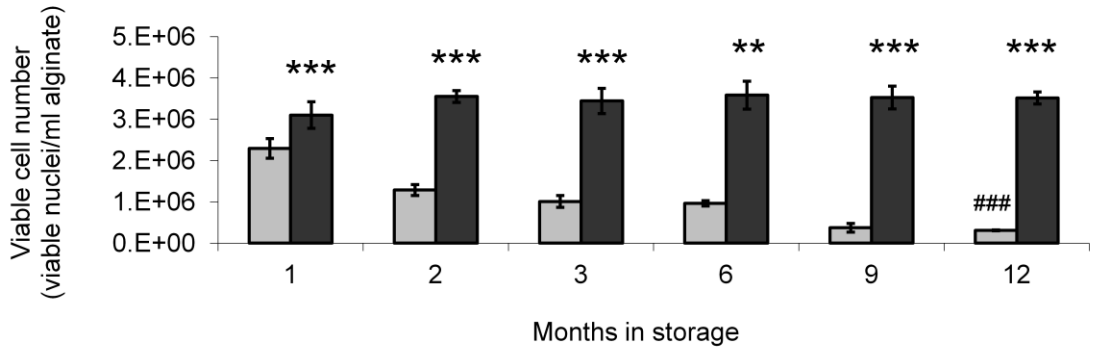


**Figure 6-14. Effect of storage temperature on ELS viable cell numbers in post-warming cultures.**

**ELS were cryopreserved in 12% DMSO/UW with cholesterol using a multi-step cooling profile (Diener et al. 1993) and stored either in the vapour phase of liquid nitrogen (-150°C) (dark grey bars) or at -80°C light grey bars). ELS were warmed rapidly using a 37°C waterbath. 24h post-warming, viable cell numbers were measured. **p<0.01, ***p<0.005 -150°C compared to -80°C at same time point. ###p<0.005 compared to 1 month storage.**

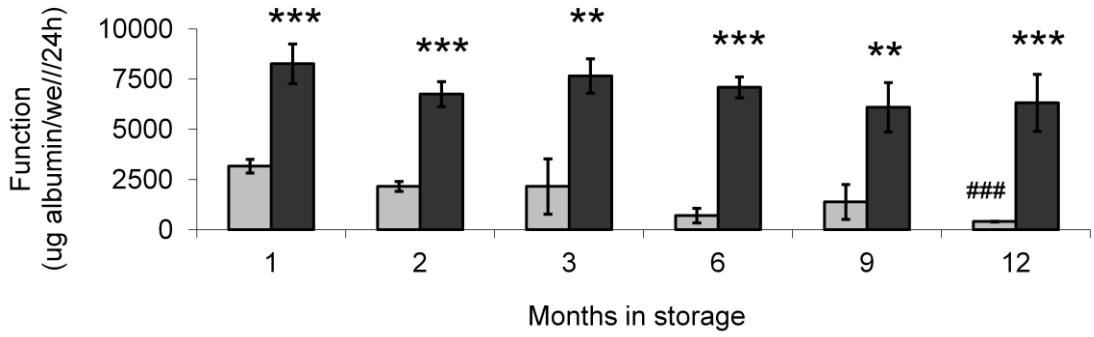


**Figure 6-15. Effect of storage temperature on ELS function in post-thaw cultures.**

**ELS were cryopreserved in 12% DMSO/UW with cholesterol using a multi-step cooling profile (Diener et al. 1993) and stored either in the vapour phase of liquid nitrogen (-150°C) (dark grey bars) or at -80°C light grey bars). ELS were warmed rapidly using a 37°C waterbath. 24h post-warming, function was measured. **p<0.01, ***p<0.005 -150°C compared to -80°C at same time point. ###p<0.005 compared to 1 month storage.**

Similarly, ELS function was maintained between 1 and 12 months for in ELS stored at -150°C. In agreement with the viable cell number data, function of ELS stored at -80°C

also decreased progressively over time to a minimum after 12 months at ~10% that of ELS stored at -150°C at the same point. Interestingly, function of ELS stored at -80°C appeared to decline more rapidly than viable cell numbers for storage periods as short as 1 month, function was already reduced to around one third that of ELS stored at -150°C. At all time points, ELS function was significantly improved by storage at -150°C cf. -80°C (Figure 6-15 on the previous page).

6.4.5.1. Determination of glass transition temperature

The modulated DSC trace (Figure 6-16 below) shows a glass transition, apparent by a slight dip in the reverse heat flow (circled in red) at approximately -120°C.

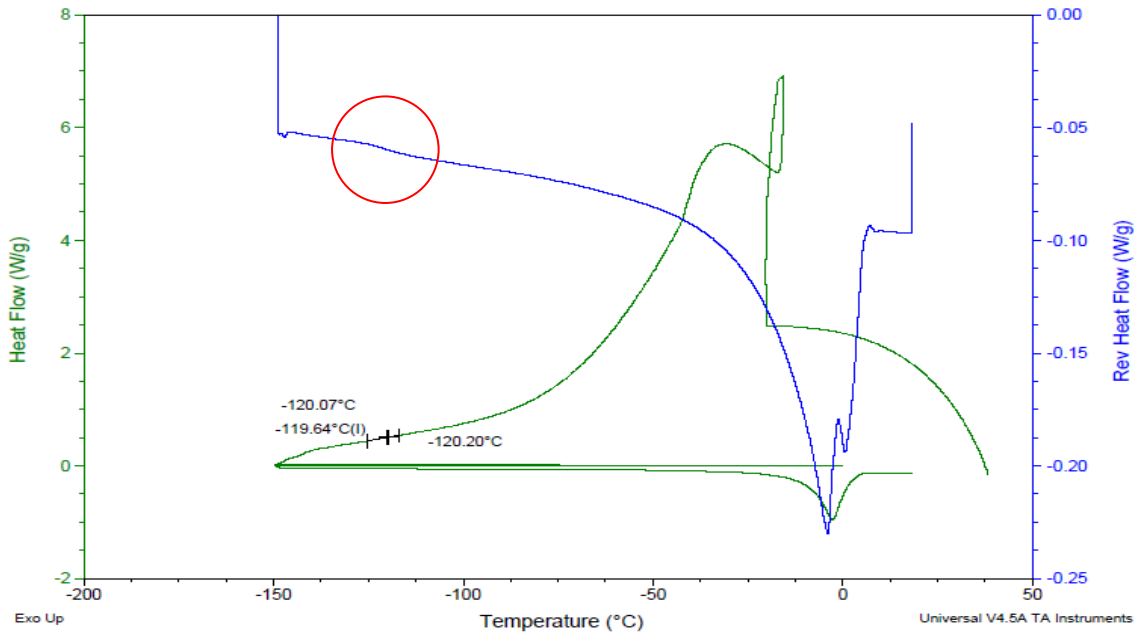


**Figure 6-16. Determination of Tg using DSC.**

**80µl aliquots of 12% DMSO in UW solution were cooled to -150°C. Samples were analysed using DSC at 5°C/min with modulation of 1°C/min. This trace is representative of 2 repeats.**

## 6.5. Discussion

The overall aim of this Chapter was to consider cryopreservation of ELS from a regulatory viewpoint.

Foetal calf serum (FCS) is commonly used in cryopreservation protocols but being of xenogeneous origin is not suitable for use here, where the intended end-application is treatment of human patients. The use of FCS could potentially pose risks of viral transmission and also invoke an immune response to animal proteins in patients where sepsis or weakened immune systems are already likely. It is difficult to ensure that FCS is completely removed from cryopreserved samples, even with washing steps and so now the use of FCS is less preferable and the more regular use of allogeneic serum is now reported. This is particularly apparent in progenitor cell cryopreservation applications including biobanking (Zeisberger et al. 2010).

Here, 10% fresh frozen plasma (FFP) has been shown to be beneficial during cryopreservation of ELS with improved viability, cell number and function. Viability was also significantly improved at 50% FFP but this is improvement is not seen in the cell number and function data indicating that this "improved" viability may be a result of dead cells degenerating and no longer existing for staining with PI, apparently increasing viability. This dose-dependent response to FFP is likely due to the beneficial components of FFP (i.e. membrane stabilisation) countered by those components which become detrimental at high concentrations as they are excluded from the ice lattice.

One point for future consideration is that HepG2 monolayer cultures are currently grown in media supplemented with 10% FCS (as described in Chapter 2) prior to encapsulation. This does not comply with current GMP requirements for regenerative medicine applications and so will need to be considered but is beyond the scope of this thesis. Commonly, stem cell cultures intended for human treatment are cultured using human serum instead of FCS and efforts are underway to avoid the use of xeno- (typical murine-) feederlayers (Unger et al. 2008). For the purposes of this section though, the use human FFP appears effective for cryopreservation of ELS in place of FCS.

The next part of this study was to reduce the concentration of DMSO required to protect ELS during cryopreservation whilst maintaining good functional recovery. DMSO is known to be cytotoxic and so its use at any stage of BAL production (including cryopreservation) is disadvantageous. The current protocol utilises 12% DMSO to which ELS are exposed to for 15 minutes on ice prior to cryopreservation. When ELS

were exposed to 12% DMSO for longer than this, a progressive decline in recovery was observed out to one hour exposure time confirming previous findings that DMSO is damaging to hepatocytes with extended exposure time (Coundouris et al. 1993).

When ELS were exposed to 5% DMSO for 15 minutes, recovery was worse than when compared to 12% DMSO. This indicates that the intracellular concentration of DMSO achieved using this method was insufficient to protect ELS. However, by increasing exposure time out to 1 hour, a progressive improvement in recovery was observed. This may indicate that a more complete permeation of DMSO throughout the spheroids was possible when sufficient time was allowed. No literature regarding rate of DMSO permeation into hepatocyte spheroids could be found but in other tissue types CPA pre-incubation times of more than 1 hour (cartilage) (Carsi et al. 2004) and up to more than 6 hours (heart valve leaflets) (Lakey et al. 2003) are recommended for complete permeation of DMSO at or close to 4°C. Clearly, the time required for DMSO to permeate is tissue- type and size dependent and should be measured in ELS to confirm whether sufficient time has been allowed. Despite the improved recovery of ELS with extended exposure time to 5% DMSO, recovery was still lower than the current optimal protocol which perhaps indicates that 12% DMSO is necessary for optimal recovery of ELS.

Raffinose, along with other sugars such as trehalose, sucrose and lactose, has previously been shown to offer protection to various cell types including sperm (Koshimoto & Mazur 2002). The use of disaccharides has also been suggested as a method of reducing the requirement of high concentrations of DMSO in cells during cryopreservation that are destined for human treatment. This was demonstrably successful for human foetal liver haematopoietic progenitor cells where DMSO concentration was reduced from 10%, to either 2 or 5% with sucrose concentrations of between 0.05M and 0.3M in addition. At both 2 and 5% DMSO, sucrose addition resulted in a dose-dependent improvement in recovery and viability. This was most apparent for 2% DMSO where recovery was approximately quadrupled when 0.3M sucrose was added and was also apparent at 5% (although the improvement was not so great) (Petrenko et al. 2008). Here though, no additional protection was conveyed by raffinose. The reasons for this are unclear but may result from differing osmotic tolerances or characteristics of ELS to progenitor cells or the fact that epithelial cells (such as ELS) are more complex,

differentiated and contain more organelles which may increase their susceptibility to injury by this mechanism.

The use of GMP-compliant preservation media during cryopreservation of ELS is preferable due to the intended end-application (i.e. treatment of human patients suffering with ALF). Recovery of ELS cryopreserved in all GMP-compliant CPA media was similar, with the exception of Profreeze where, admittedly small, but nevertheless statistically significant, improvements in recovery were observed.

No studies could be found directly comparing UW to any of the (cryo)preservation media used here but UW is consistently cited as the optimal vehicle solution for both cryopreservation and temporary hypothermic storage of primary liver cells both as spheroids (Lee et al. 2004), or as single cell suspensions (Janssen et al. 2004; Kunieda et al. 2003). Here, there were no significant differences in recovery between ELS cryopreserved in UW and the other vehicle solutions (except Profreeze) regardless of whether they are intended as organ preservation solutions or as cryopreservation media, indicating that the constituents are likely to be similar.

Additionally, or alternatively, ELS may already be sufficiently tolerant to stresses that specific additives in these media are intended to ameliorate. For example, previous investigations have found that mRNA coding for some heat shock proteins is increased in 3D cultures (Choudhury et al. 2003). Much of the literature focuses on primary cell types as this technology is more commonly used for preservation of whole organs, whereas HepG2 cells comprising ELS are a cell line. This may account for the perceived insensitivity of ELS to subtle differences between the compositions of the media trialled here. Furthermore, here ELS were exposed to the media only briefly (15 minutes) before cryopreservation which is far shorter than typical exposure times for hypothermic storage of organs where studies may continue for up to 2 days before differences become apparent (Janssen et al. 2004).

Despite this, within this thesis (Chapter 4) it has been previously demonstrated that oxidative stress, for example, occurs as a result of hypothermia (Vreugdenhil et al. 1991) independently of ice nucleation, that will occur later during cryopreservation suggesting that the concentrations and mechanisms of protection of the antioxidants contained within UW may be comparable to those contained within the other media trialled here. Therefore, by adding DMSO to UW to protect ELS from damage resulting from ice formation, similar recoveries are observed. Unfortunately however,

this is somewhat speculative as the exact constituents of the commercial cryopreservation media are unknown.

Similarly, it is difficult to suggest reasons for the improved recovery observed using Profreeze as its constituents are unknown. Profreeze is commercially available from Lonza at a cost approximately twice that of UW solution ("Profreeze" 2011), considered the gold standard for hepatocyte preservation and cryopreservation in this thesis. However, as cryopreservation of ELS in large volumes will eventually be required for a BAL, whether the small improvement in observed functional recovery of ELS in post-thaw cultures justifies the increased cost (which would be in the region of a few thousand pounds per BAL) is debatable. This argument is further compounded by the data from Chapter 4 where UW solution can be improved by the addition of antioxidants and growth factors, suggesting that the use UW solution for cryopreservation of ELS is adequate here.

To produce an 'off-the-shelf' BAL, cryopreservation of ELS will be required. Liquid nitrogen as a cryogen in cooling machines, is the current gold standard, and has been used for a number of years despite issues of both sterility and safety. Today, regulatory authorities are keen on cell therapies being deliverable without the use of liquid nitrogen at any point of the cold chain as this would overcome the safety concerns of using liquid nitrogen or other cryogens. Therefore, an alternative cryogen-free cooling system, the EF600, based upon a Stirling cryocooler, was evaluated for cryopreservation of ELS.

As described in Chapter 3, cryopreservation of ELS is complex due to the need to ensure intracellular ice formation is avoided. Cooling rates in the region of 1-2°C/min have been shown to allow sufficient time for individual hepatocytes to dehydrate with good functional recovery. These slow cooling rates were successfully applied here using the EF600.

However, as described in Chapter 3, ELS are very sensitive to supercooling even in small amounts. The protocol utilised for this comparison includes a shock cooling step, which the EF600 was unable to perform. As an alternative method of avoiding supercooling and inducing nucleation of water to form ice, cholesterol was included in both cohorts of cryopreserved cells to minimise supercooling and subsequent cryo-injury resulting from intracellular ice formation and propagation thereafter. This approach was successful in both the Kryo10 and in the EF600.

Cryogen-based CRFs rely on removal of heat from the sample by convection, and the multi-step cooling profile used was designed within, and for, such a system. Conversely, the EF600 removes heat from the sample by conducting heat away from the sample to the metal cooling plate. When temperatures were measured at the top and bottom of the vials, a large temperature gradient was apparent and heat removal from the top of the vial was not achieved.

Furthermore, when cholesterol was included as a nucleator in 1.25ml volumes, the samples supercooled by around 5°C (likely sufficient to cause significant cryo-injury). Cholesterol floats on the surface of the liquid and so it was hypothesised that the cholesterol was only in contact with the liquid above the equilibrium melting point (i.e. too warm to allow nucleation to occur). However, given that the sample temperature at the bottom of the vial was below the equilibrium temperature, by inverting the cryovials and mixing the "cold" and "warm" portions of the sample, the overall sample temperature was reduced to below the equilibrium melting point and so cholesterol triggered nucleation of the supercooled liquid to ice.

Once this effect was recognised, the sample volume within each vial was reduced from 1.25ml to 500µl in order that the entire sample be cooled more evenly and cholesterol able to act as a nucleator. These differing methods of heat transfer (i.e. conductive vs. convective) did impact on the cooling profiles observed by the samples but by modifying the protocol slightly, these effects were ameliorated.

For cells cryopreserved with either the cryogen-based CRF or the Stirling engine-based CRF, high viabilities were seen on rewarming and are likely to be attributed at least in part to these approaches to ensure control of ice nucleation. To make a direct comparison between traditional liquid nitrogen-cooling systems and this new cryogen-free cooling system, ELS were cryopreserved using identical cryoprotectants, cooling profiles and cryovials, but in either the (cryogen-cooled) Kryo10 or the (Stirling engine-cooled) EF600. Following storage in the vapour phase of liquid nitrogen, ELS were assessed for viability, total cell numbers and function. Good recovery, as assessed using all 3 indicators of success, was achieved in both cohorts of cryopreserved cells with only one statistical difference between the two cryopreserved groups seen, in the cell numbers at 48 hours, perhaps representing a slight delay in recovery for ELS cryopreserved using the EF600 compared to those from the Kryo10 machine.

To summarise, efficacy of this cryogen-free cooling equipment for cryopreservation of encapsulated liver spheroids using a complex multi-step cooling profile has been demonstrated here. The EF600 has previously been used to cryopreserve other multicellular structures (8-cell mouse embryos) using a multi-step cooling profile and recovery of these embryos did not differ statistically significantly from those embryos cryopreserved using a nitrogen-cooled CRF (Morris et al. 2006) in agreement with the findings of this study with ELS.

Avoiding the use of nitrogen entirely during both cooling and storage is favourable. In the previous section, it was demonstrated that the cooling profile may be applied using a cryogen-free cooler. However, the ELS utilised for these experiments were stored in the vapour phase of liquid nitrogen. Although storage in the vapour phase is preferable to storage in the liquid phase in terms of contamination risk, ideally cryogens should be avoided entirely. Cryogen-free freezers (to simply maintain a set low temperature) do exist and some have the capability to store samples at very low cryogenic temperatures but are extremely costly, are not routinely available and often rely on a nitrogen back-up. Nonetheless, storage in a standard -80°C freezer was compared to storage in the vapour phase of liquid nitrogen (approximately -150°C) over a period of one year.

Viability, cell number and function were all assessed at 24 hours post-warming as this is when the effects of latent injury from cryopreservation are fully apparent (see Chapter 3). Moreover, as this was a prospective study, ELS were cryopreserved in a single batch and so inter-batch variation is not relevant for this study and any differences apparent result from the different storage temperatures used. ELS stored below -150°C in the vapour phase of liquid nitrogen maintained viability, cell number and function over the year. This indicates that, as expected, these ELS were stored at a sufficiently low temperature to prevent degradation. These findings are in agreement with other studies investigating the effect of long-term storage at or close to -150°C of various cell and tissue types including sperm (Yogev et al. 2010) and cardiac valves (Mirabet et al. 2008) where no loss of viability or function was observed during long-term storage, in some instances for as long as 28 years (Clarke et al. 2006).

However, ELS stored at -80°C showed significantly reduced viability, cell number and function when compared to those stored at -150°C after as little as one month. Thereafter, the recovery of ELS stored at -80°C worsened with extended storage time, indicating a strong negative correlation between storage time and ELS recovery. Only

one study could be found that prospectively compares storage of samples (aortic valves) at -80°C with storage at -150°C. In agreement with the findings here, they found that storage at -80°C was comparable to that at -170°C after 2 weeks but thereafter, functional recovery of those stored at -80°C fell and after 8 weeks storage, cells were non-functional (Feng et al. 1996). Similarly, haematopoietic progenitor cells stored at -80°C began to lose functional recovery capabilities beyond 6 months storage and beyond approximately 2 years, function was not recoverable (Galmes et al. 1999). In another study, cryopreserved porcine hepatocytes stored at -80°C showed decreased viability and functional capability (ammonia removal) after only 10 days storage which declined over time so that after 5 months storage function was reduced by at least 50% depending on the cryopreservation media used (Kunieda et al. 2003). In a separate study, adult human hepatocytes lost approximately 15% viability over 14 days storage at -80°C (Coundouris et al. 1993).

Evidently storage at -80°C is not sufficiently cold to prevent degradation of ELS, which means that some "mobile" fraction is conserved even at this low temperature. The DSC trace shows that a glass transition is present at approximately -120°C which is very close to that quoted in another similar study where a similar CPA mix was used (Pegg et al. 1997). The glass transition represents the temperature below which the amorphous material (likely to be freeze-concentrated) is no longer viscous but becomes brittle and immobile. This could account for the loss of recovery of ELS stored at -80°C but not at -150°C. In this study, the -80°C ELS were never cooled to below -80°C so were never cooled below the glass transition temperature. It is possible that if the ELS had been cooled to below $T_g$, the degradation of the sample may not have been so apparent. However, devitrification or recrystallisation of the freeze-concentrated fraction would still likely occur above -120°C as ELS warmed when transferred to -80°C for long-term storage.

As some groups have reported that -80°C is sufficient for long-term storage (although this work shows that ELS cannot be stored at this temperature) this is not necessarily the case for all cell types and cryopreservation protocols. Glass transition temperatures will vary between samples (due to different CPAs utilised) so this could account for the variations reported in the Introduction to this Chapter. Alternatively, different cell types or culture formats may possess capabilities to cope with these injury mechanisms better.

For example, if recrystallisation happens to mechanically disrupt cell-cell contacts within ELS, this may amplify the damaging effect of recrystallisation.

Generally, it seems samples should be stored below or as close to the glass transition temperature as is possible. This will normally involve the use of liquid nitrogen unless a Stirling engine-based freezer is available that is capable of maintaining temperatures below -130°C. This is likely to be at significant cost and further testing would be required to assess whether long-term storage within such a freezer was appropriate before it could be recommended for long-term storage of ELS.

## 6.6. Conclusions

The aim of this chapter was to consider cryopreservation of encapsulated liver cell spheroids from a regulatory perspective. As the BAL is intended for treatment of human patients suffering from acute liver failure, mandates described by regulatory bodies both in Europe and the US must be complied with. As this is only one aspect of this thesis, only some points have been considered but these are not exhaustive and further consideration would need to be given before the BAL could enter clinical use.

Firstly, the use of xenogeneous material is unacceptable. Here, FFP has been demonstrated to offer protection from cryopreservation, optimally at a concentration of 10%. This means that FCS need not be included within the CPA media thus the risk invoking an immune response is reduced and regulatory requirements are met.

Secondly, different GMP-compliant organ preservation and cryopreservation media were trialled. There were no significant differences between the media with the exception of Profreeze. ELS do not appear to be sensitive to the use of different vehicle media and so the use of less expensive UW solution appears to be adequate here.

Thirdly, although the use of DMSO is not prohibited, it would be desirable to reduce the concentration of DMSO used during cryopreservation as it has known cytotoxic effects. Here, it was hypothesised that by using a lower concentration of DMSO and either increasing incubation time and/or including a non-penetrating CPA, similar recoveries may be observed. Unfortunately this was not the case and it seems that 12% DMSO must be used for successful recovery of ELS following cryopreservation.

Finally, recently introduced regulations state that any nitrogen used should be of pharmaceutical grade which is extremely costly. The use of nitrogen also presents safety hazards and risk of cross contamination between stored samples. If cryopreservation could be achieved without nitrogen this would be advantageous. Here it was demonstrated that cryopreservation of ELS using a cryogen-free cooler is possible with comparable recovery to that of ELS cryopreserved using a nitrogen-based cooler. However, for long-term storage of ELS, nitrogen is required to ensure that samples are stored below the glass transition of the CPA mixture. It may be possible to store ELS in cryogen-free freezers set to below -130°C but this has not been tested here.

As stated previously, this is not an exhaustive list of considerations but demonstrates that strategies can be developed to adapt optimised cryopreservation protocols to meet regulations as required.

# CHAPTER 7

# Cryopreservation of Primary Human Hepatocytes

## 7.1. Introduction

The aim of this thesis was to develop and optimise a cryopreservation protocol for encapsulated liver cell spheroids (ELS) as the cellular component of a bioartificial liver machine. This has been relatively successful and some specific additions to the standard cryopreservation media (12% v/v dimethylsulfoxide in University of Wisconsin solution) have been demonstrated to be useful in improving recovery of ELS following cryopreservation.

These additives have different mechanisms of action that result in improved recovery of ELS (containing HepG2 cells) following cryopreservation: preventing supercooling to reduce likelihood of intracellular ice formation (cholesterol); protecting against apoptosis (1K1); preventing oxidative stress (Trolox and catalase); and plasma membrane stabilisation and osmotic buffer (fresh frozen plasma). However, it was unknown whether or not these additives could protect primary human hepatocytes (PHH) from the damaging effects of cryopreservation. PHH represent the gold standard as cells expressing the full range of hepato-specific functions, but may be a more sensitive cell population to identify improved cryopreservation strategies. In addition, successful cryopreservation of PHH could provide a valuable research model for studies of xenobiotic metabolism and toxicology. Furthermore, PHH are potentially an alternate source of cells for a BAL although they are more likely to be utilised as a whole organ for transplantation in patients suffering acute liver failure, rather than being used for PHH production.

### 7.1.1. How well do PHH fare following cryopreservation using standard techniques?

Animal hepatocytes could substitute PHH for *in vitro* models, but inter-species variations exist, highlighting the need for successful cryopreservation of PHH (Li et al. 2009; O'Brien et al. 2004). Similarly, human cell lines typically lack the level and repertoire of cytochrome P450 function required to accurately model PHH (with the possible exception of HepaRG cell line) (Guillouzo et al. 2007).

The potential of PHH has not yet been fully recognised due, in part, to a shortage of available cells. However, if PHH could be successfully cryopreserved, this would allow PHH to be banked and thawed (and pooled if required) as necessary. Many have attempted cryopreservation of PHH with varying success and whilst some report development of improved protocols (Terry et al. 2005), others are less optimistic (Stephenne et al. 2010), suggesting that function is too low in cryopreserved PHH to be of use for transplant or to be representative of fresh PHH for *in vitro* models.

As described earlier in this thesis, some of these differences are due to the time following cryopreservation at which recovery is measured and the methods used to measure recovery. However, an additional factor is that of PHH source in the first place. Cells are likely to be sourced from either resected tissue (as here) or from non-heart beating donors where the liver has been rejected for transplant due to anatomical abnormality or it is unsuitable for another reason (for example, too fatty or cirrhotic). Here it was not possible to select age or sex of donor but all patients underwent resection following secondary metastases of colon cancer and all PHH were isolated from the normal liver tissue after removal of the tumour using the same methodology which has been shown to affect PHH recovery following cryopreservation (Terry et al. 2005).

### 7.1.2. Can additives to the CPA mixture improve recovery following cryopreservation?

In Chapter 3, cholesterol was demonstrated to act as a heterogeneous nucleator, enabling ice formation at temperatures close to the equilibrium melting point (close to the highest temperature at which ice crystals can exist). This in turn, is likely to reduce the potential for intracellular ice formation within ELS resulting in a much improved cryopreservation protocol. ELS are susceptible to the effects of supercooling but PHH have also been demonstrated to be damaged by supercooling (Diener et al. 1993). Non-linear profiles have been shown to result in improved recovery (Diener et al. 1993; Terry et al. 2006). However, even with this protocol, recovery of ELS was improved further using cholesterol. Therefore this approach was trialled here for cryopreservation of PHH.

In Chapter 4, 1K1 was demonstrated to protect ELS from apoptosis during cryopreservation. 1K1 is an engineered version of hepatocyte growth factor (HGF) and has been demonstrated to be effective at protecting PHH previously, but when apoptosis

was triggered using Fas ligand, not as a result of the stresses associated with cryopreservation (Ross et al. 2009). It was hypothesised that 1K1 could protect PHH from apoptosis caused by cryopreservation (as for ELS) and result in improved recovery.

Also in Chapter 4, it was demonstrated that ELS suffer from oxidative stress which could be reduced using antioxidants (Trolox and catalase). PHH have also been shown to suffer from oxidative stress (Illouz et al. 2008) following isolation and cryopreservation and some have reported that improved viability can be obtained in post-thaw cultures when antioxidants are included during cryopreservation (Galbiati et al. 2010; Sasnoor et al. 2005). It was hypothesised that post-cryopreservation recovery of PHH could also be improved using catalase and Trolox.

In Chapter 6, FFP was demonstrated to improve recovery of ELS from cryopreservation optimally at 10%. It seems likely that FFP may also protect PHH from the damaging effects of cryopreservation too and so this was trialled here.

Whilst the effect of fructose on ELS during cryopreservation has not been investigated, it has been demonstrated to be successful for cryopreservation of PHH by others. The reasons that the authors suggest for its efficacy are well-aligned with the injury mechanisms described in this chapter (i.e. it protects against oxidative stress and simply against apoptosis) {Terry, 2006 423 /id}. Therefore, it was hypothesised that fructose may protect PHH from damaging effects of cryopreservation, resulting in improved recovery in post-thaw cultures.

Having established that cholesterol, 1K1, antioxidants, FFP and fructose are beneficial additions to CPA media for cryopreservation of PHH, it was hypothesised that these may have a synergistic effect. Although some are likely to protect using mechanisms that overlap, it was hypothesised that some cumulative effect would be observed.

## 7.2. Aims

The specific aims of this chapter were to investigate whether or not the same additions that improve recovery of ELS also improve recovery of PHH. The data from previous chapters suggests that individual additives are likely to result in incremental increases in liver cell recovery. However, when combined, it was hypothesised that the additives would have a synergistic effect. Therefore, in this chapter, PHH were cryopreserved with or without individual additives and then with or without all the additives in combination and recovery in post-thaw cultures assessed using a variety of assays.

## 7.3. Methods

### 7.3.1. Isolation and Culture of PHH from Liver Samples

Liver tissue samples were obtained from the Surgical Department at the North Hampshire Hospital, Basingstoke, UK from patients undergoing surgery for metastases of colon cancer to the liver. All patients gave informed consent for the use of resected tissue for research, as approved by the Research and Ethics Committee of the hospital. Throughout this chapter each sample has been assigned the prefix BS followed by a number to indicate which isolation data refers to.

Primary epithelial cells (i.e. PHH) present additional challenges to an epithelial cell line (i.e. HepG2 cells) in that they must first be isolated from whole liver prior to culture. Moreover, they are known not to proliferate well in culture, rapidly lose their differentiated function, and require extracellular matrix for adhesion to a tissue culture treated surface and so were cultured on collagen coated plates here.

#### 7.3.1.1. Isolation of PHH from Liver Samples

Materials

Chelating Buffer (20mM HEPES, 0.5mM EGTA in PBS)

Perfusion Buffer (20mM HEPES in PBS)

Digestion Buffer (20mM HEPES, 1.5g BSA (0.5% w/v), 50µg/ml ascorbic acid and 4µg/ml insulin in 300ml HBSS with calcium and magnesium. Immediately prior to digestion of the liver 150mg Collagenase Type IV (0.05% w/v) and 30mg DNase I (0.01%w/v) were added

Dispersal Buffer (50ml (10%v/v) FCS and 50mg DNase I (0.01% w/v) in 500ml Williams' Medium E (WEM))

2% Trypan blue in PBS

Haemocytometer

Method

Primary adult human hepatocytes were isolated from freshly resected liver samples by 2 step collagenase perfusion and differential centrifugation in line with previously published methods (Saich et al. 2007). This was performed by other colleagues.

Cell number and viability was determined using trypan blue exclusion of non-viable cells. 160µl of HBSS, 20µl of trypan blue and 20µl of homogeneous cell suspension

were mixed and incubated for 2 minutes. 9µl of this solution was loaded onto a haemocytometer chamber and both viable and non-viable cells were counted.

Donors were of either sex with ages ranging between 40 and 75 undergoing resection for liver tumours. Cell viability determined using trypan blue exclusion was between 46% and 77% when measured immediately post-isolation. The full donor information is given in Table 7-1 below.

**Table 7-1. Tissue donor information.**

| Liver | Sex | Age | Viability post-isolation |
|---|---|---|---|
| BS89 | F | 50 | 55% |
| BS90 | M | 75 | 55% |
| BS92 | M | 59 | 61% |
| BS93 | F | 40 | 46% |
| BS94 | M | 65 | 60% |
| BS95 | M | 58 | 77% |

7.3.1.2. Culture of Isolated PHH

*7.3.1.2.1. Preparation of Collagen Type I from Rat Tail Tendons*

Materials

Rat tails

0.01M acetic acid

500ml conical flask

Magnetic stirrer

50ml polypropylene centrifuge tubes

Centrifuge

Sterile universal tubes

Method

Rat tails were sterilised using ethanol and transferred to a class II microbiological safety hood. Under sterile conditions, using artery clamps, the vertebrae of the tail from tip to base were sequentially broken. The central tendon was removed after each break and total tendon removed weighed. 300ml acetic acid was added to each gram of tendon in a conical flask. The mixture was stirred for 72 hours at 4°C until the tendon had dissolved. This solution was transferred to 50ml polypropylene centrifuge tubes and centrifuged for 2 hours at 800g. Supernatant was dispensed into sterile universal tubes and stored at 4°C until required.

*7.3.1.2.2. Collagen Coating of Cell Culture Plates*

Materials

24-, 48- and 96-well tissue culture plates

Sterile HBSS without calcium and magnesium

Sterile saline

Collagen type I (prepared from rat tails as above)

UV lamp

Method

Collagen was pipetted down the side of individual wells and incubated for 5 minutes at room temperature. Following this, excess collagen solution was vigorously flicked from the wells before 2 rinses with HBSS. Sterile saline solution was added to each well and the plates were irradiated without the lid under UV light for 15 minutes. Plates were stored at 4°C until required and were irradiated for 15 minutes again immediately before use. The volumes of collagen solution, HBSS and sterile saline solution added for each plate size are given below in Table 7-2.

**Table 7-2. Volumes of collagen solution, HBSS and saline solution to be added for each plate size.**

| Plate Size | Collagen solution (ml) | HBSS (ml) | Sterile saline solution (ml) |
|---|---|---|---|
| 24-well | 0.5 | 1 | 1 |
| 48-well | 0.2 | 0.5 | 0.5 |
| 96-well | 0.1 | 0.25 | 0.25 |

*7.3.1.2.3. Preparation of PHH Culture Medium (PHH Medium)*

Materials

Phenol Red-Free Williams' Medium E (WEM) (Sigma W4128)

10% Foetal calf serum

2mM L-Glutamine

100U/ml Penicillin/0.1mg/ml Streptomycin

1.25µg/ml Fungizone

10nM Dexamethasone

Method

For media preparation, all supplements, except foetal calf serum (FCS), were pooled into a 50ml centrifuge tube and 0.2µm filter-sterilised prior to addition to culture media.

*7.3.1.2.4. Culture of PHH*

Cell culture work was performed in a class II microbiological safety cabinet using disposable sterile plastic ware. PHH were normally plated at the following densities, unless otherwise stated:

24-well plate: $0.5x10^6$ per well

48-well plate: $0.25x10^6$ per well

96-well plate: $0.1x10^6$ per well

PHH were left to attach overnight and were washed twice with warmed culture medium the following day to remove any non-viable cells. Warmed medium was used to replenish spent culture medium. PHH were cultured at 37°C within a humidified atmosphere with 95% air, 5% $CO_2$ in an incubator.

### 7.3.2. Cryopreservation of PHH

Materials

DMSO

UW solution

WEM

1.8ml cryovials (Nunc)

Centrifuge tubes

Kryo 10 CRF

37°C water bath

Centrifuge

Method

Isolated PHH were resuspended at a concentration of $2x10^7$ cells/ml in WEM. A 20% v/v DMSO/UW mixture (unless otherwise stated) was prepared and cooled on ice. The PHH cell suspension was mixed 1:1 with the DMSO/UW mixture on ice (to give a final DMSO concentration of 10% and final cell concentration of $1x10^7$ cells/ml) and 1ml well-mixed aliquots transferred into cryovials. PHH were cryopreserved using a non-linear cooling profile (Diener et al. 2003) in the Kryo 10 freezer and transferred to the vapour phase of liquid nitrogen for storage. PHH were thawed rapidly using a 37°C water bath. DMSO was diluted out slowly using iced WEM over 12 minutes. PHH were centrifuged at 400rpm (50g) (without brake) for 6 minutes, before supernatant was removed and replaced with PHH medium. PHH were diluted further if required before plating onto collagen-coated tissue culture plates.

### 7.3.3. Cryopreservation with additives

Materials

2.2mg/ml cholesterol in 20% DMSO/UW
200nM 1K1 in 20% DMSO/UW
20000IU/ml catalase in 50mM phosphate buffer (pH 7.4) (40x stock)
3.4mM Trolox and 1000IU/ml catalase in 20% DMSO/UW
20% FFP in 20% DMSO/UW
600mM fructose in 20% DMSO/UW
WEM
1.8ml cryovials (Nunc)
Centrifuge tubes
Kryo 10 CRF
37°C water bath
Centrifuge

Method

PHH were cryopreserved using a method identical to that above in section 7.2.2 except that instead of mixing PHH 1:1 with 20% DMSO in UW, PHH were mixed with 1:1 20% DMSO in UW containing the additive/s to be tested.

### 7.3.4. Assays to assess recovery in post-warming cultures

Once PHH had been cryopreserved, recovery was assessed out to 72 hours post-warming and results compared to unfrozen PHH at equivalent time point using a variety of assays, described below.

#### 7.3.4.1. Viability assay

MTT was utilised to assay PHH metabolic viability here. Fluorescent microscopy was not used as the population of PHH is not homogeneous (both before and after cryopreservation) and so by using MTT a better representation of PHH recovery (as a whole population) was achieved.

Materials

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma M5655)
Sterile PBS
4mM HCl in isopropanol
96-well clear flat bottomed plates

Plate sealer

Orbital plate shaker

Spectrophotometer at 570nm

Method

0.75mg/ml stocks of MTT were prepared in sterile PBS and stored at -20°C in single use aliquots until required. PHH were washed twice with sterile PBS and an appropriate volume of pre-warmed MTT added to each well of cells (500µl for a 24-well plate). PHH were incubated in a humidified 37°C 5% $CO_2$ incubator for 3 hours until blue/purple crystals had formed. After this time, MTT substrate was aspirated and an appropriate volume of acidified isopropanol was added to each well. Plates were sealed and shaken using an orbital plate shaker for 30 minutes until all crystals had dissolved. Supernatant was then transferred to a new 96-well plate and absorbance measured at 570nm.

7.3.4.2. Attachment assay

Unlike ELS, PHH could be assayed for attachment post-cryopreservation. This was achieved by quantifying total DNA from both attached and unattached PHH. Attachment to substratum is one global assessment of viability, because the attachment process requires normal metabolic activity and ultrastructural organisation. DNA was quantified using Hoechst dye 33342 which binds to AT rich regions in the minor groove of the DNA duplex. Hoechst dye can be excited and fluorescence emitted measured; concentration of DNA present in samples calculated by comparison to a DNA standard curve.

Materials

Collagen-coated 48-well plates

PHH media

Sterile PBS

0.1M NaOH

1M Tris HCl (pH 7)

0.1M TNE buffer – (Tris base, EDTA, NaCl)

1M Tris HCl Buffer

TNE Buffer (10mM Trisbase (pH7.4), 0.2M NaCl, 1mM EDTA) (pH 7.4)

1mg/ml Hoechst dye in TNE buffer (1000x stock)

25µg/ml Calf thymus DNA in 1:1 mixture of 0.1M NaOH:1M Tris HCl (pH 7)

2ml microfuge tubes

Microfuge

-20°C freezer

96-well clear flat-bottomed plates

Aluminium foil

CytoFluor

Method

PHH were left to attach overnight in 48-well plates. The next day, PHH media (containing unattached PHH) was collected into microfuge tubes. Attached PHH were washed a further 2 times with sterile PBS and both times aspirated media collected and added to microfuge tubes. Plates were stored at -20°C until the assay was performed. PHH unattached in the washes were centrifuged at 14000rpm for 4 minutes at 4°C. Supernatant was removed and replaced with sterile PBS. PHH were centrifuged once more and supernatant discarded and cell pellets stored at -20°C until assayed for DNA content.

800µl 0.1M NaOH was added to each microfuge tube. 500µl 0.1M NaOH was added to each well, swirled and contents transferred to a new microfuge tube. A further 300µl was added to each well, swirled and contents added to the microfuge tube. 800µl 1M Tris HCl was added to each microfuge tube and 100µl of this transferred to individual wells of a 96-well plate.

A calf thymus DNA standard was prepared from 25µg/ml to 0.390625µg/ml by serial dilution with a 1:1 mixture of 0.1M NaOH and 1M Tris HCl. 100µl of each standard was transferred to individual wells of a 96-well plate and a diluent blank included. 1000x Hoechst dye stock was diluted to 1x using TNE buffer and 100µl added to each well (the plate was protected from light at all times).

Fluorescences of each sample and standard were measured in duplicate using a fluorescent plate reader (360/480 excitation/emission) using 100 reads per well. DNA concentration of both attached and unattached PHH was calculated from the standard curve on each plate. Attachment was then calculated and expressed as a percentage as below:

$$attachment\,(\%) = \frac{DNA from attached PHH}{DNA from attached PHH + DNA from unattached PHH} x100$$

7.3.4.3. Functional assays

*7.3.4.3.1. Hepato-specific protein synthesis and secretion*

ELISAs were used to quantify secretion of hepato-specific proteins from PHH using the methods described in Chapter 2. However, as FFP, which includes relatively high concentrations of albumin, was sometimes included within cryopreservation media, and because PHH do not produce AFP, PHH could only be assayed reliably for production of fibrinogen, alpha-1-antitrypsin or prothrombin.

*7.3.4.3.2. Cytochrome P450 activity*

Broad-spectrum CPY450 activity was measured using the methods described in Chapter 2. PHH were induced using indirubin for 48 hours prior to assay.

### 7.3.5. Apoptosis assay

In addition, PHH from one liver were assayed for apoptosis using the caspase GLO assay.

Materials

96-well clear-bottomed plates

96-well white-walled plates

Serum-free PHH media

Caspase GLO reagent (Promega G8090)

Luminometer

Method

PHH were plated at a cell density of 40,000 cells per well in 96-well plates in a total volume of 100µl serum-free PHH media. 100µl caspase GLO reagent was added to each well and mixed and incubated for 1 hour in the dark at room temperature. After this time, 100µl of this mixture was transferred to individual wells of a white-walled 96-well plate and luminescence measured. Appropriate blanks were included and deducted from each reading.

### 7.3.6. Lipid peroxidation assay

In addition, PHH from one liver were assayed for lipid peroxidation 5h post-plating using the malondialdehde assay as described in Chapter 2. Due to large differences in attachment efficiency between cohorts, results were normalised to total protein content from PHH that remained attached after washing.

## 7.4. Results

### 7.4.1. Characterisation of fresh PHH

#### 7.4.1.1. Inter-sample variation

Unlike HepG2 cells, populations of PHH are likely to be heterogeneous both between donors and within individual donors. Indeed, the digestion process itself used to isolate PHH can also introduce variation between samples. Inter-donor variation was large and is most easily seen by looking at micrographs of PHH which show differing levels of attachment (despite plating at similar densities) and morphology (see Figure 7-1 below). In some instances, PHH flattened quickly displaying a cuboidal morphology, similar to that, which would be seen *in situ*. However, this was not always true and in other instances, PHH remained "rounded up". For this reason, an unfrozen control was always included for all PHH cryopreservation experiments such that inter-donor variation was accounted for when interpreting the results.

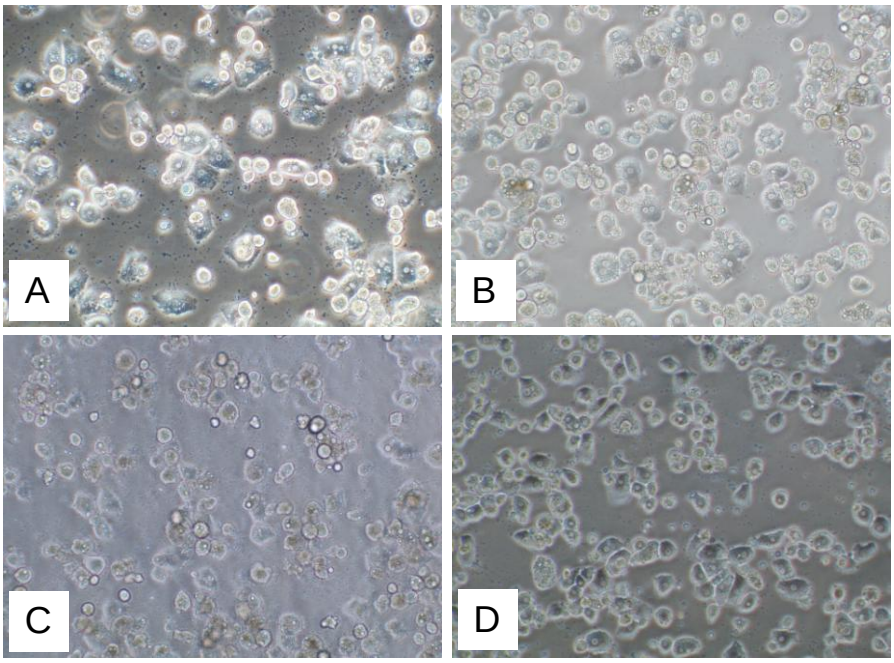


**Figure 7-1. Typical hepatocyte morphology and attachment 18h post-isolation.**

**Examples of different hepatocyte cultures following overnight attachment after isolation from whole liver (A-PHH 90, B-PHH 92, C-PHH 95, D-PHH 97). Both attachment and morphology differ between donors, as do the number of cell-cell contacts formed. Original magnification x20.**

### 7.4.2. Comparison of fresh PHH and PHH cryopreserved using standard CPA medium

#### 7.4.2.1. Attachment

Attachment of freshly isolated PHH and PHH cryopreserved using the standard method was measured after overnight attachment. Average attachment of freshly isolated PHH was 50.1% (from 4 separate livers) but ranged from 35.4% to 78.3%. When these cells were cryopreserved using the standard method of cryopreservation, average attachment was significantly reduced to 19.7% but ranged from 12.1% to 39.2%. Over the 4 livers, on average, attachment of unfrozen PHH was 0.4-fold that of fresh PHH from the same liver but again variation was large and ranged between 0.2- and 0.9-fold.

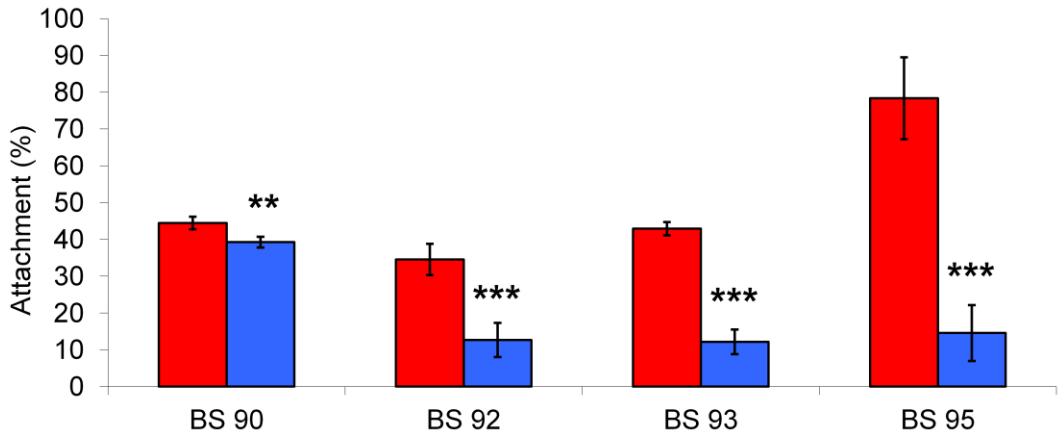


**Figure 7-2. Attachment of fresh and cryopreserved PHH after overnight culture.**

**Attachment of fresh (red bars) and cryopreserved (blue bars) are expressed as a percentage. Cryopreserved PHH were cooled in 12% DMSO/UW using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Results from 4 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. **p<0.005, ***p<0.001 compared to fresh PHH from the same liver at equivalent time point.**

#### 7.4.2.2. MTT Reduction

Metabolic viability measured using MTT reduction was measured in fresh PHH and PHH cryopreserved using the standard method over 3 days following plating. MTT reduction in fresh PHH fell over time with continued culture but was variable between livers. However, at all time points, MTT reduction was significantly greater in fresh PHH compared to cryopreserved PHH in all livers at approximately 0.09-fold, 0.07-fold and 0.08-fold that of fresh PHH at 24, 48 and 72h post-plating respectively (Figure 7-3).

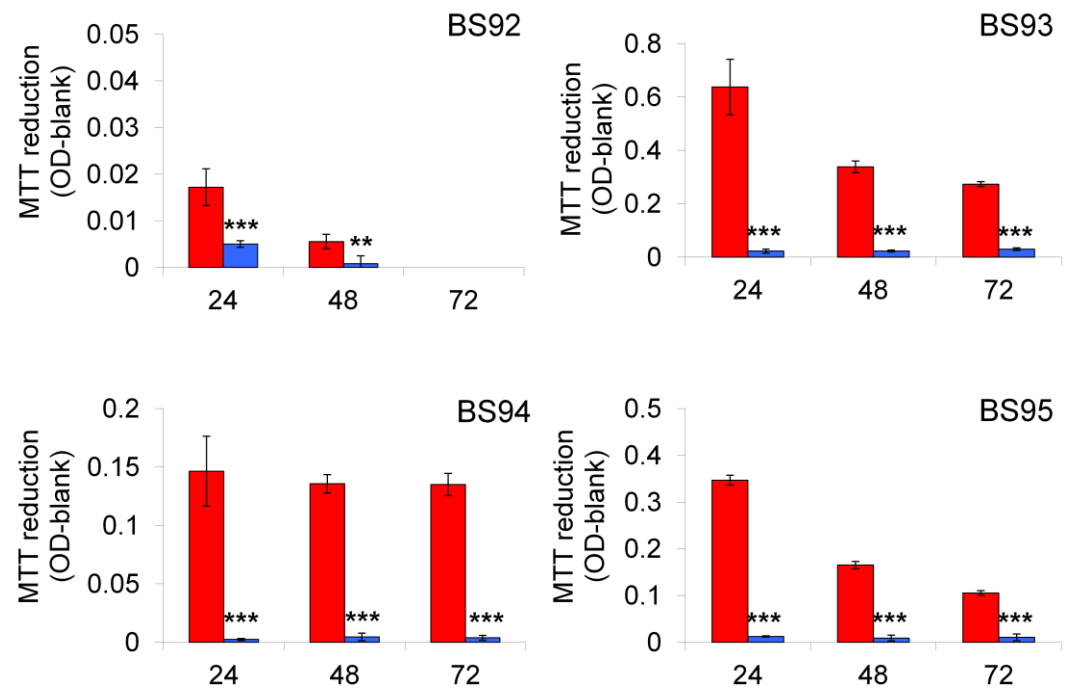


**Figure 7-3. MTT reduction of fresh and cryopreserved PHH in post-warming cultures.**

**Comparison of MTT reduction between fresh (red bars) and cryopreserved (blue bars) PHH up to 72 hours post-plating. Cryopreserved PHH were cooled in 12% DMSO/UW using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of assay is indicated on the x axis. Results from 4 separate livers are shown. MTT reduction was not measured in BS92 after 72 hours. Data from individual livers are averages of n=5 wells +/- SD. **p<0.005, ***p<0.001 compared to fresh PHH from the same liver at the equivalent time point.**

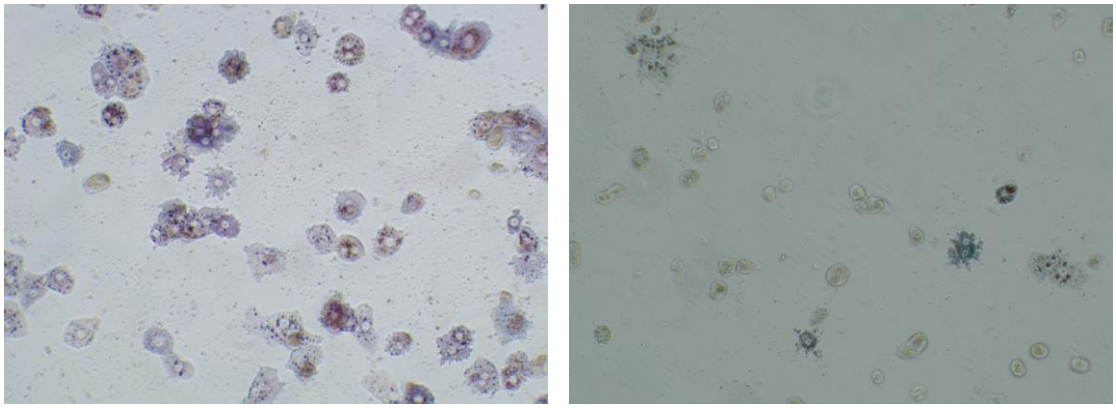

**Figure 7-4. Micrographs showing MTT substrate reduction in fresh and cryopreserved PHH.**

**Comparison of MTT reduction between fresh (left) and cryopreserved (right) PHH 72 hours post-plating. MTT reduction product appears as blue/purple crystals. Original magnification x20.**

When images of PHH assayed using MTT were captured 72 hours post-plating, nearly all fresh PHH had produced the blue/purple crystal product. However, this was not true

of cryopreserved PHH where only some attached cells produced the MTT reduction product (Figure 7-4).

### 7.4.2.3. Protein synthesis and secretion

Protein synthesis and secretion was measured using ELISAs in fresh PHH and PHH cryopreserved using the standard method over 3 days following plating. Protein synthesis and secretion was variable between livers. However, at all time points measured protein synthesis and secretion was significantly greater in fresh PHH compared to cryopreserved PHH in both livers.

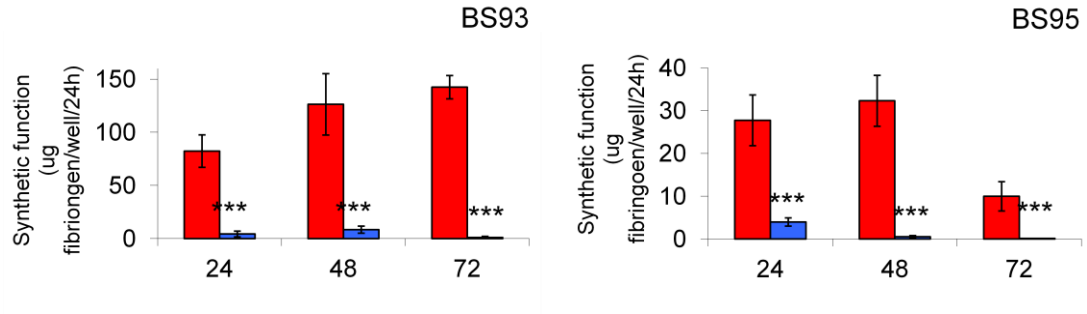


**Figure 7-5. Synthetic function of fresh and cryopreserved PHH in post-warming cultures.**

**Comparison of protein synthesis and secretion between fresh (red bars) and cryopreserved (blue bars) PHH up to 72 hours post-plating. Cryopreserved PHH were cooled in 12% DMSO/UW using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of assay is indicated on the x axis. Results from 2 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. ***p<0.001 compared to fresh PHH from the same liver at the equivalent time point.**

### 7.4.2.4. Cytochrome P450 activity

Broad spectrum CYP450 function was measured using ECOD assay in fresh PHH and PHH cryopreserved using the standard method at 48 and 72 hours post-plating (after 48 hour chemical induction using indirubin). CYP450 function fell in both fresh and cryopreserved PHH with time. Cryopreserved PHH showed significantly reduced CYP function at both time points measured at less than 1% of equivalent fresh PHH (Figure 7-6).

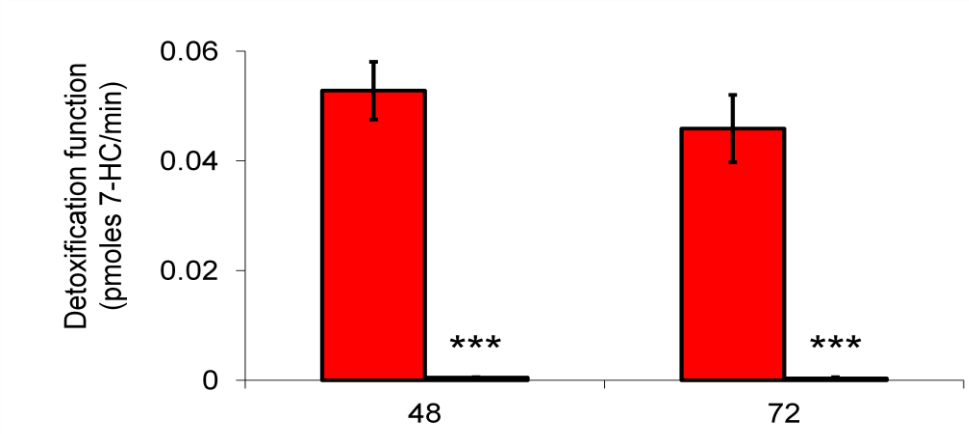


**Figure 7-6. Detoxification function of fresh and cryopreserved PHH in post-warming cultures.**

**Comparison of CYP450 function between fresh (red bars) and cryopreserved (blue bars) PHH at 48 and 72 hours post-plating following 48 hour chemical induction. Cryopreserved PHH were cooled in 12% DMSO/UW using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD. ***p<0.001 compared to fresh PHH at the equivalent time point.**

### 7.4.3. Effect of cholesterol during cryopreservation

#### 7.4.3.1. Attachment

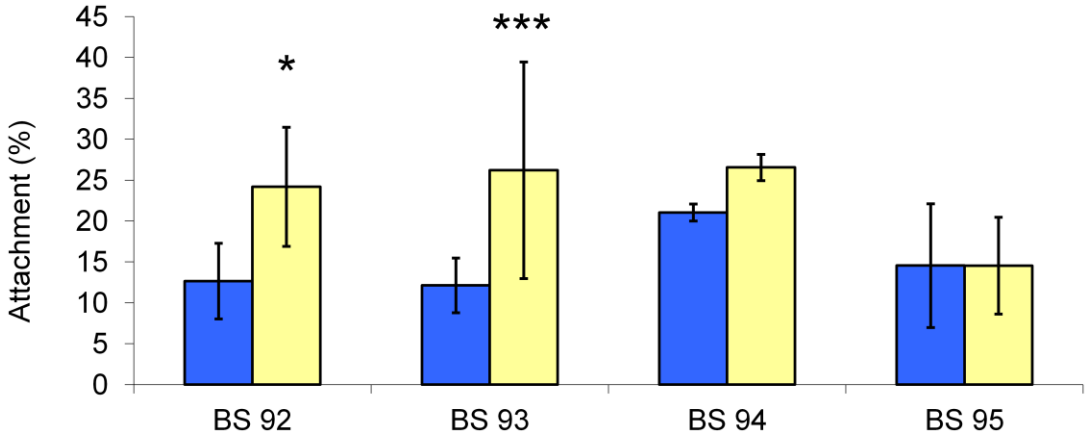


**Figure 7-7. Attachment of PHH cryopreserved with or without cholesterol.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (yellow bars) cholesterol using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Results from 4 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, ***p<0.001 compared to PHH cryopreserved using the standard CPA media at the same time point.**

Average attachment of PHH cryopreserved using the standard cryopreservation method was 15% (14%, 12%, 13% and 21% from individual livers) but was improved to an average of 21% (14%, 24%, 26% and 27% from individual livers). In 3 of the 4 livers, attachment was increased by varying amounts using cholesterol (although this was only statistically significant in 2 donor samples). In the fourth liver, cholesterol did not affect attachment efficiency (Figure 7-7 on the previous page).

### 7.4.3.2. MTT reduction

In 2 of the 4 livers, MTT reduction was unaffected by the inclusion of cholesterol during cryopreservation. In the other 2 livers, MTT reduction was improved at all time points measured when cholesterol was included during cryopreservation, although this was not always statistically significant and was only slight in one of the livers.

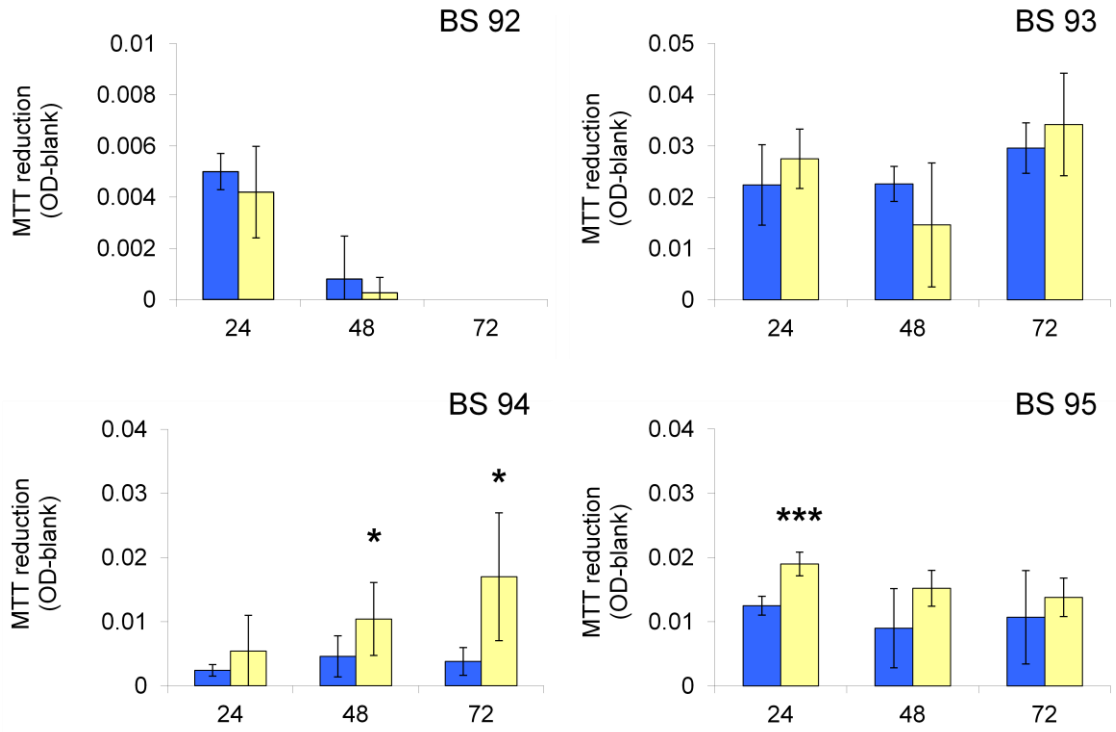


**Figure 7-8. MTT reduction of PHH cryopreserved with or without cholesterol in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (yellow bars) cholesterol using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. MTT assays were performed as indicated on the x axis. Results from 4 separate livers are shown. MTT reduction was not measured in BS92 after 72 hours. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, ***p<0.001 compared to PHH from the same liver cryopreserved using standard CPA media at the same time point.**

### 7.4.3.3. Protein synthesis and secretion

Protein synthesis and secretion was increased at all time points measured when cholesterol was included during cryopreservation cf. standard cryopreservation. This was statistically significant at both 48 and 72 hours post-cryopreservation.

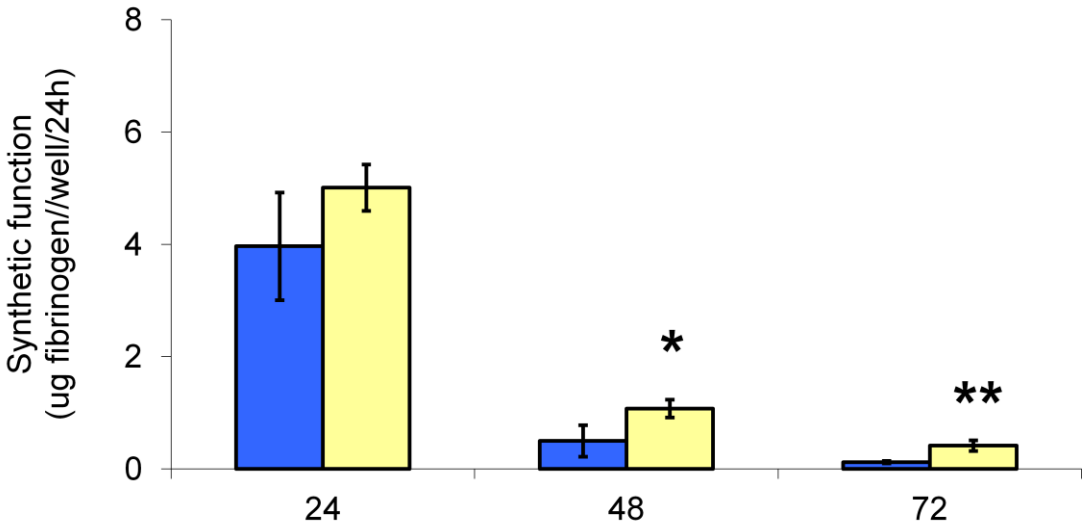


**Figure 7-9. Synthetic function of PHH cryopreserved with or without cholesterol in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (yellow bars) cholesterol using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of function assay is indicated on the x axis. Results from 1 liver (BS 95) are shown. Data are averages of n=5 wells +/- SD. *p<0.05, **p<0.01 compared PHH from the same liver cryopreserved using standard CPA media at the same point.**

### 7.4.3.4. Cytochrome P450 activity

Cytochrome P450 activity was significantly improved at both 48 and 72 hours post-cryopreservation (following 48-hour chemical induction with indirubin) when PHH were cryopreserved using cholesterol-modified CPA media cf. standard CPA media. At both times, CYP450 activity was approximately doubled in cholesterol-modified CPA media cf. standard CPA media (Figure 7-10).

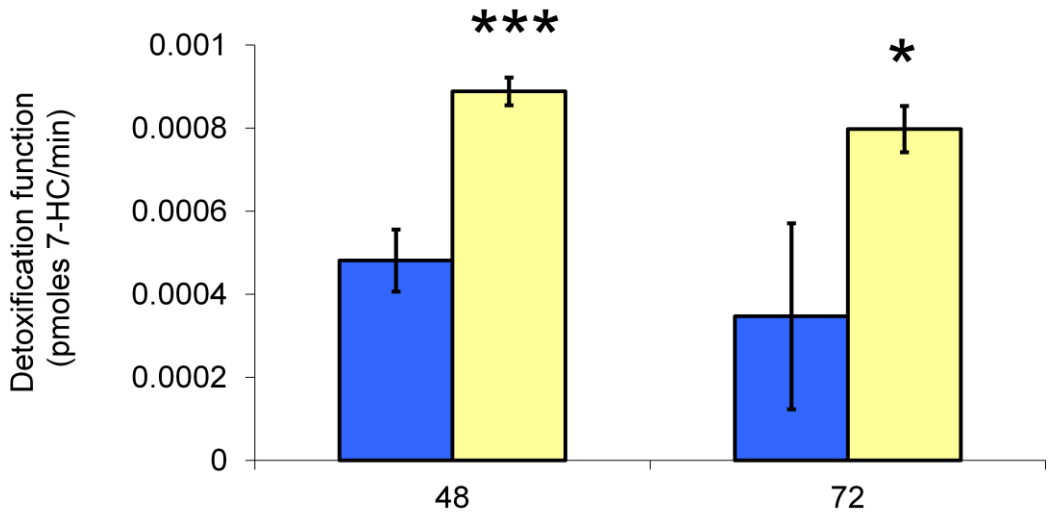


**Figure 7-10. Detoxification function of PHH cryopreserved with or without cholesterol in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (yellow bars) cholesterol using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. CYP450 function was measured at 48 and 72 hours post-plating following 48 hour chemical induction. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD. *p<0.05, ***p<0.005 compared to PHH from the same liver cryopreserved using standard CPA media at the same time point.**

### 7.4.4. Effect of 1K1 during cryopreservation

#### 7.4.4.1. Attachment

PHH attachment was improved by the inclusion of 1K1 during cryopreservation in all 4 livers, although the improvement was only statistically significant in 2 of the livers. Average attachment without 1K1 was 15% (ranging from 12% to 21%) but improved to an average of 21% (ranging from 16% to 26%) with 1K1 (Figure 7-11).

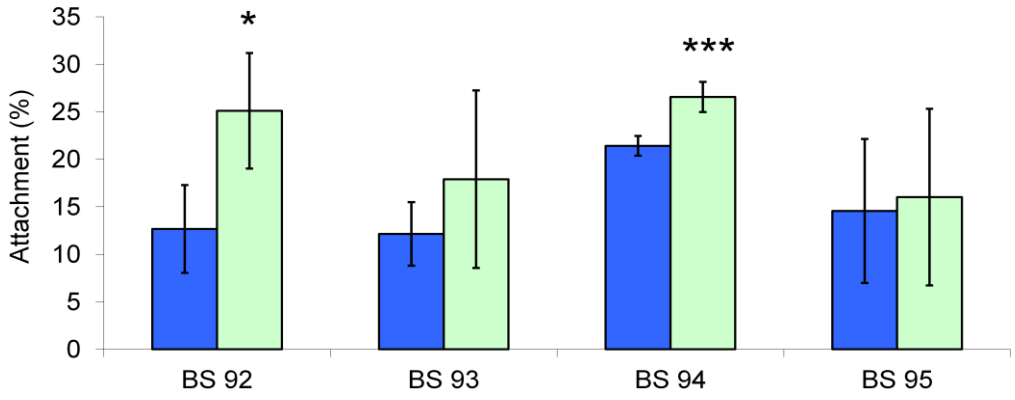


**Figure 7-11. Attachment of PHH cryopreserved with or without 1K1.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (green bars) 1K1 using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Attachment was compared after overnight attachment. Results from 4 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, ***p<0.001 compared to PHH from the same liver cryopreserved using standard CPA media at the same time point.**

### 7.4.4.2. MTT Reduction

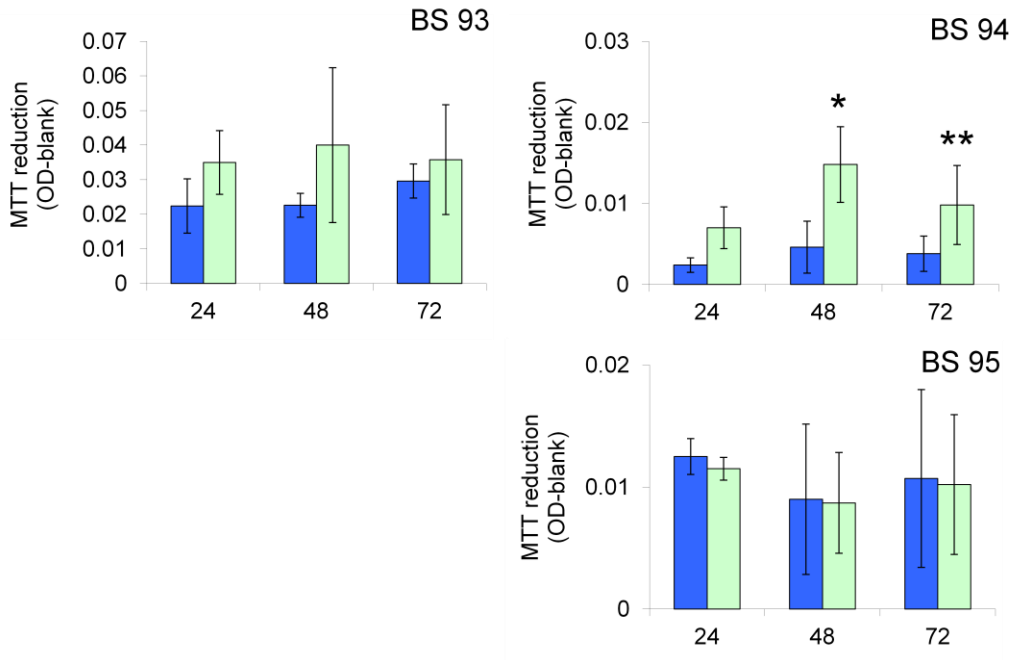


**Figure 7-12. MTT reduction of PHH cryopreserved with or without 1K1 in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (green bars) 1K1 using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of MTT assay is indicated on the x axis. Results from 3 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, **p<0.01 compared to PHH from the same liver cryopreserved using standard CPA media at the same time point.**

MTT reduction was increased in 2 of the 3 livers at all time points measured although this was only significant in one of the livers at 48 and 72 hours post-plating. In the third liver, 1K1 did not affect MTT reduction (Figure 7-12 on the previous page).

### 7.4.4.3. Protein synthesis and secretion

Protein synthesis and secretion was approximately doubled at all time points when 1K1 was included during cryopreservation although this was not statistically significant at any time point measured.

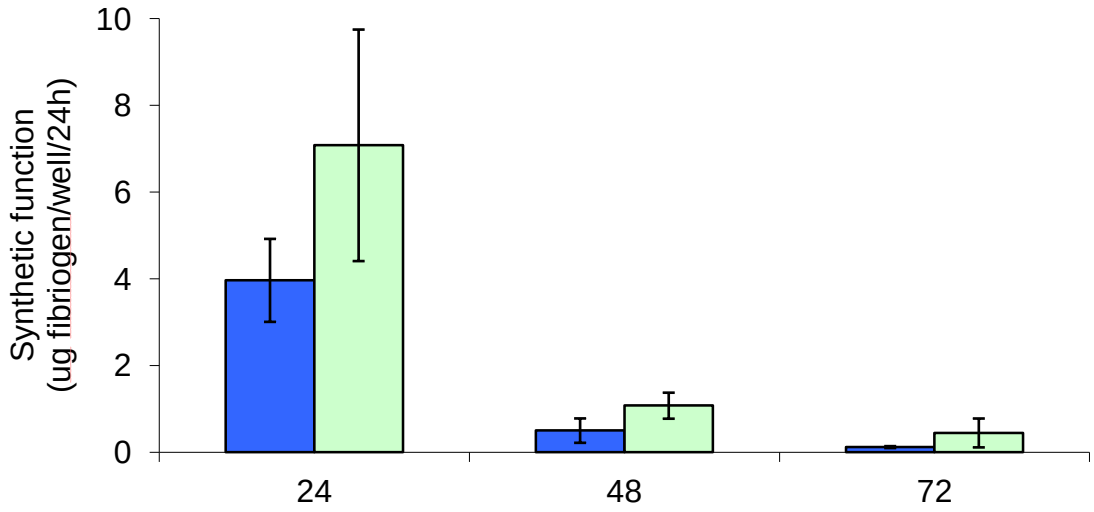


**Figure 7-13. Synthetic function of PHH cryopreserved with or without 1K1 in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (green bars) 1K1 using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of function assay is indicated on the x axis. Results from 1 liver (BS 95) are shown. Data are averages of n=5 wells +/- SD.**

### 7.4.4.4. Cytochrome P450 activity

Cytochrome P450 activity was not affected by the inclusion of 1K1 during cryopreservation (Figure 7-14).

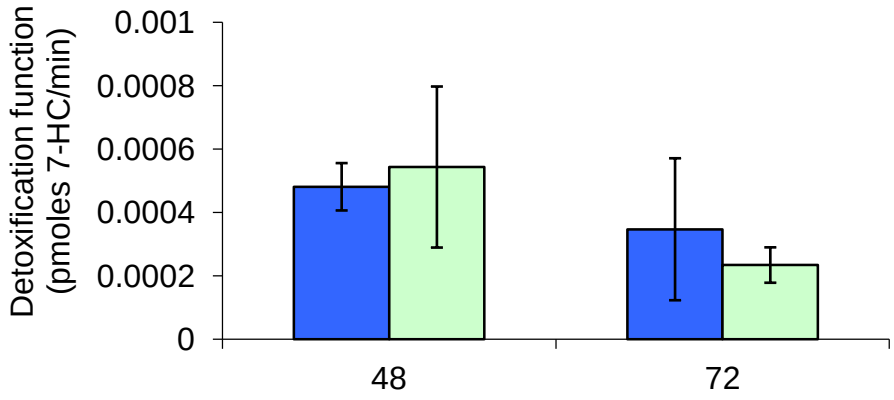


**Figure 7-14. Detoxification function of PHH cryopreserved with or without 1K1 in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (green bars) 1K1 using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. CYP450 function was measured at 48 and 72 hours post-plating following 48 hour chemical induction. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD.**

### 7.4.4.5. Apoptosis

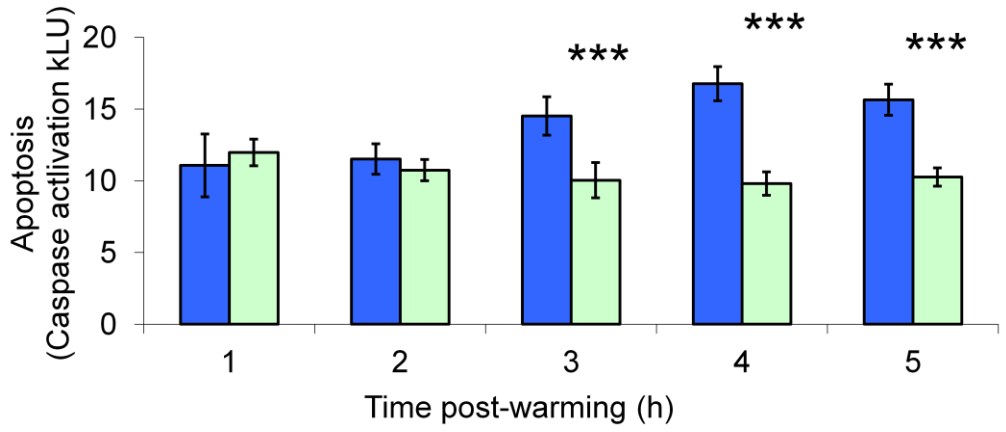


**Figure 7-15. Apoptosis of PHH cryopreserved with or without 1K1 in the first 5 hours post-warming.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (green bars) 1K1 using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Caspase activation was measured out to 5 hours post-warming. Results from 1 liver (BS93) are shown. Data are averages of n=5 wells +/- SD. ***p<0.005 compared to PHH cryopreserved using standard CPA media at the same time point.**

Apoptosis in cryopreserved PHH was measured hourly out to 5h post-plating. In PHH cryopreserved using the standard cryopreservation method, caspase activation

(indicating onset of an apoptotic response) was observed beyond 3h post-plating. When 1K1 was included in the cryopreservation media, no evidence of caspase activation was observed and at 3, 4 and 5h post-plating, caspase activation was significantly reduced using 1K1.

### 7.4.5. Effect of antioxidants during cryopreservation

#### 7.4.5.1. Attachment

Attachment was almost doubled when antioxidants were included in the cryopreservation media cf. standard CPA media in one liver (BS 95), although this was not statistically significant.

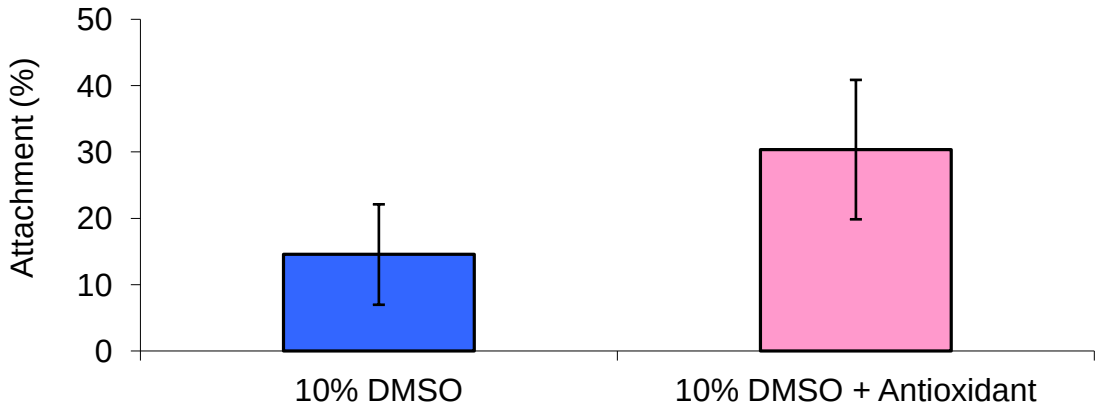


**Figure 7-16. Attachment of PHH cryopreserved with or without antioxidants.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (pink bars) antioxidants using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Attachment was measured after overnight attachment. Results from 1 liver, BS 95, are shown. Data are averages of n=5 wells +/- SD.**

#### 7.4.5.2. MTT Reduction

There was a slight trend towards improvement in MTT reduction in one of the livers by inclusion of antioxidants during cryopreservation at all 3 time points measured, but this was never statistically significant. In the second liver, MTT reduction appeared unaffected by presence of antioxidants (Figure 7-17).

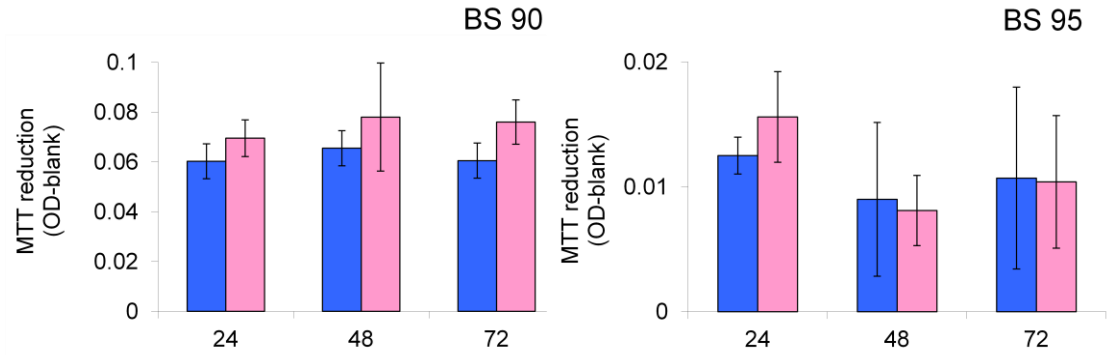


**Figure 7-17. MTT reduction of PHH cryopreserved with or without antioxidants in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (pink bars) antioxidants using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of MTT assay is indicated on the x axis. Results from 2 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD.**

### 7.4.5.3. Protein synthesis and secretion

Protein synthesis and secretion was improved at all time points measured when antioxidants were included in the CPA mixture (non significantly at 24h).

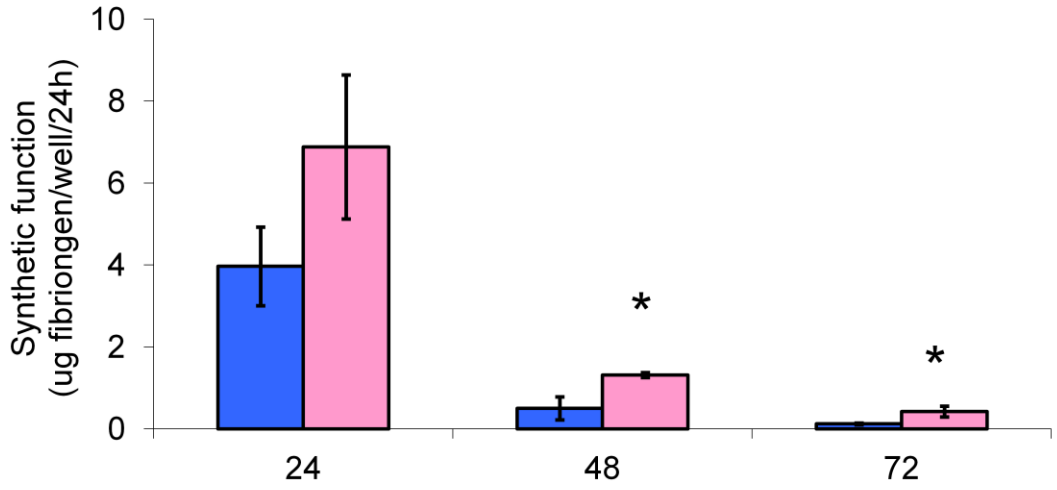


**Figure 7-18. Synthetic function of PHH cryopreserved with or without antioxidants in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (pink bars) antioxidants using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of function assay is indicated on the x axis. Results from 1 liver (BS 95) are shown. Data are averages of n=5 wells +/- SD. *p<0.05 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

### 7.4.5.4. Cytochrome P450 activity

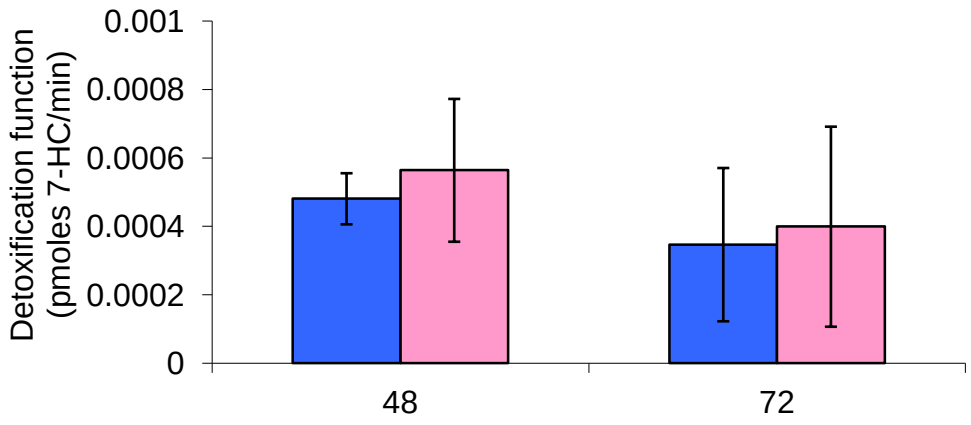


**Figure 7-19. Detoxification function of PHH cryopreserved with or without antioxidants in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (pink bars) antioxidants using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. CYP450 function assay was at 48 and 72 hours post-plating following 48 hour chemical induction. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD.**

Cytochrome P450 activity was unaffected by the inclusion of antioxidants during cryopreservation.

### 7.4.5.5. Lipid peroxidation

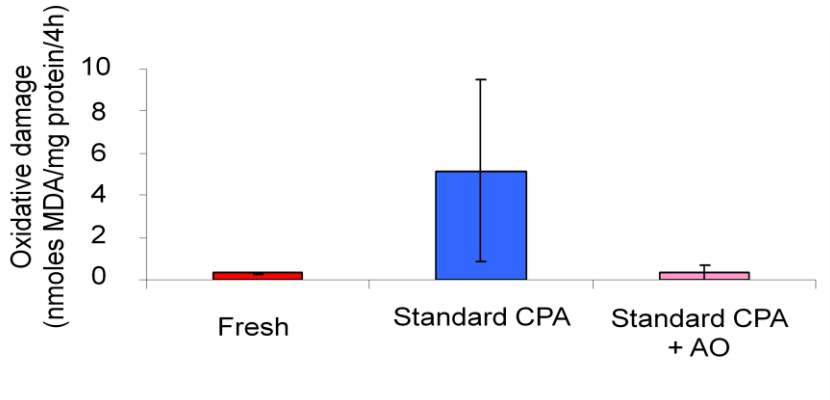


**Figure 7-20. Lipid peroxidation in PHH following cryopreservation with or without antioxidants.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (pink bars) antioxidants using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of lipid peroxidation between fresh PHH (red bar) and PHH cryopreserved using standard and antioxidant-modified CPA media at 5 hours post-plating. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD.**

Lipid peroxidation was increased by more than 10-fold in PHH cryopreserved using the standard CPA media cf. both fresh PHH and PHH cryopreserved using antioxidant-modified CPA media. However, this increase was not statistically significant. There was also no significant difference between lipid peroxidation in fresh PHH and PHH cryopreserved with antioxidant-modified CPA media (Figure 7-20 on the previous page).

### 7.4.6. Effect of FFP during cryopreservation

#### 7.4.6.1. Attachment

Attachment was significantly improved in all 4 livers when FFP was included during cryopreservation by varying amounts. Without FFP, average attachment of PHH was 13% but this was improved to an average of 41% when FFP was present during cryopreservation.

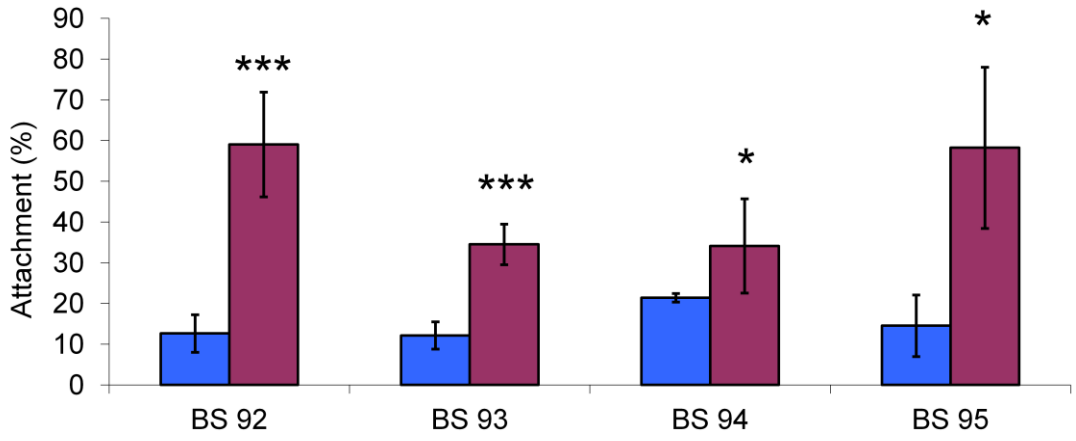


**Figure 7-21. Attachment of PHH cryopreserved with or without FFP.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (purple bars) FFP using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of attachment was made after overnight attachment. Results from 4 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, ***p<0.001 compared to PHH from the same liver cryopreserved using standard CPA media at the same time point.**

#### 7.4.6.2. MTT Reduction

MTT reduction was generally increased when FFP was included as part of CPA media. In BS 90 and BS 93, this improvement was only statistically significant at 24h post-cryopreservation. For BS 94 and BS 95, larger improvements in MTT reduction were apparent and MTT reduction was at least doubled at all time points measured for these

livers (although this was not statistically significant in BS 95 at 72 hours post-cryopreservation) (Figure 7-22).

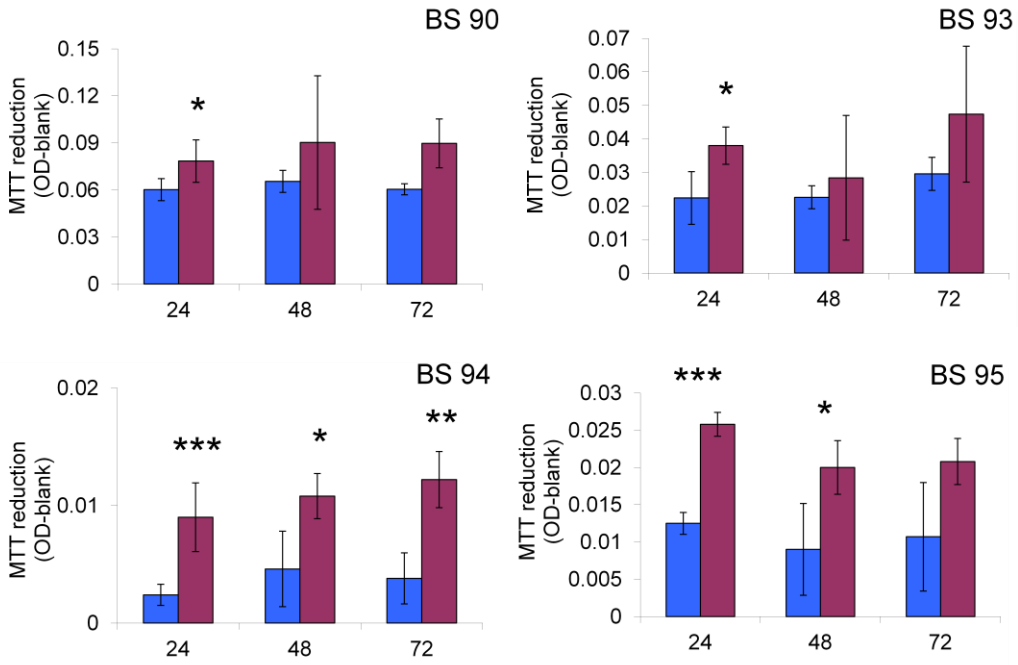


**Figure 7-22. MTT reduction of PHH cryopreserved with or without FFP in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (purple bars) FFP using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of MTT assay is indicated on the x axis. Results from 4 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, **p<0.01 and ***p<0.005 when compared to PHH from the same liver cryopreserved using standard CPA media at the same time point.**

### 7.4.6.3. Protein synthesis and secretion

Protein synthesis and secretion was improved at all time points measured by approximately 2-fold when FFP was included during cryopreservation when compared to standard CPA media (10% DMSO/UW). These improvements were statistically significant at 24 and 72 hours post-warming, but not at 48 hours post-warming (Figure 7-23).

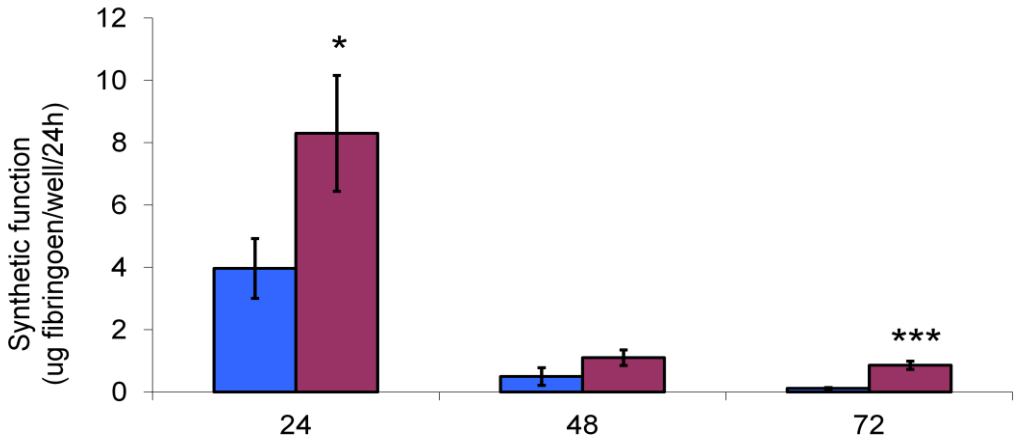


**Figure 7-23. Synthetic function of PHH cryopreserved with or without FFP in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (purple bars) FFP using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of function assay is indicated on the x axis. Results from 1 liver (BS 95) are shown. Data are averages of n=5 wells +/- SD. *p<0.05, ***p<0.005 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

### 7.4.6.4. Cytochrome P450 Activity

Cytochrome P450 activity was unaffected by FFP at either 48 or 72 hours post-plating (Figure 7-24).

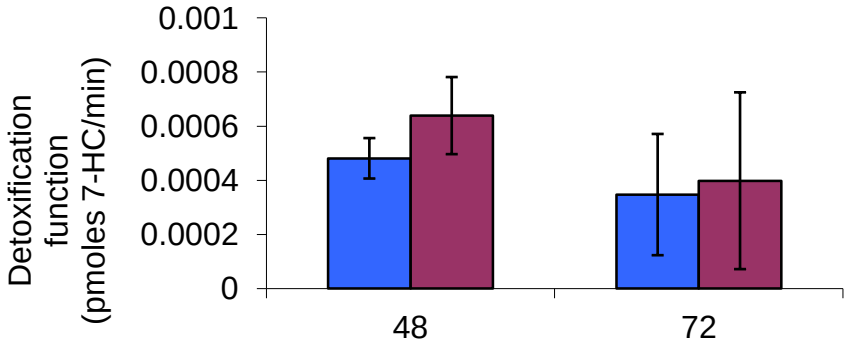


**Figure 7-24. Detoxification function of PHH cryopreserved with or without FFP in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (purple bars) FFP using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of CYP450 function between PHH cryopreserved using standard and FFP-modified was at 48 and 72 hours post-plating following 48 hour chemical induction. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD.**

### 7.4.7. Effect of fructose during PHH

#### 7.4.7.1. Attachment

Attachment efficiency was improved when fructose was included in the CPA media by 6% but this was not statistically significant.

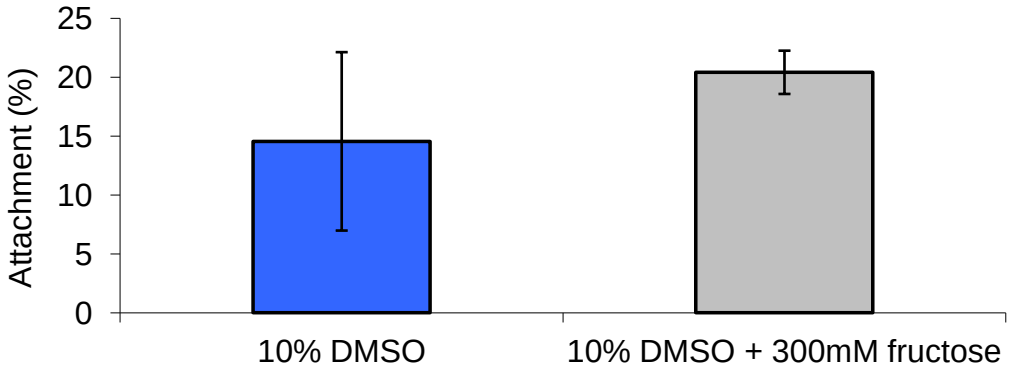


**Figure 7-25. Attachment of PHH cryopreserved with or without fructose.**

**Comparison of attachment between PHH cryopreserved using the standard method and PHH cryopreserved with 300mM fructose after overnight attachment. Results from 1 liver, BS 95, are shown. Data are averages of n=5 wells +/- SD.**

#### 7.4.7.2. MTT Reduction

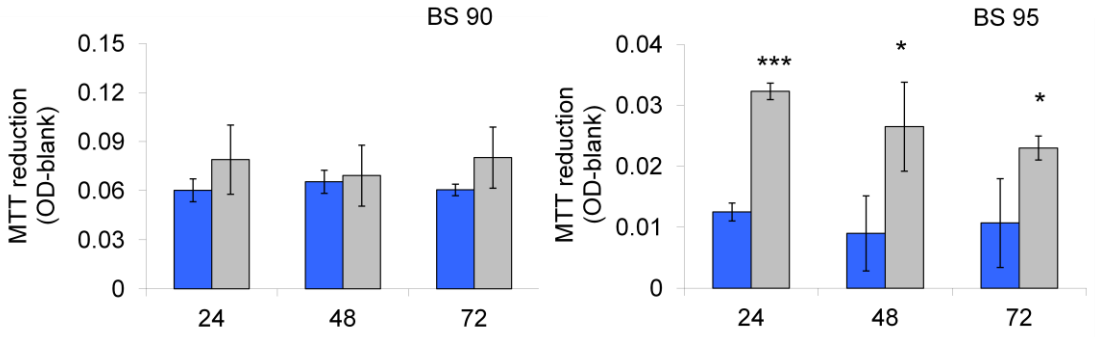


**Figure 7-26. MTT reduction of PHH cryopreserved with or without fructose in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (grey bars) fructose using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Time of MTT assay is indicated on the x axis. Results from 2 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. *p<0.05, ***p<0.005 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

The effect of including fructose within CPA media on MTT reduction was measured in 2 livers: BS90 and BS95; and results between the 2 livers were very different. In BS 90, small and statistically insignificant improvements in MTT reduction were observed

at all 3 time points. Conversely, in BS95, large (up to 3-fold) improvements were seen at all time points when fructose was included during cryopreservation. However, the total activity was very low in BS95, as was improvement, and so did not reach levels of BS90.

7.4.7.3. Protein synthesis and secretion

Protein synthesis and secretion was improved at all time points measured when fructose was added to the CPA media, by approximately 2-fold. This was statistically significant at both 48 and 72 hours post-warming.

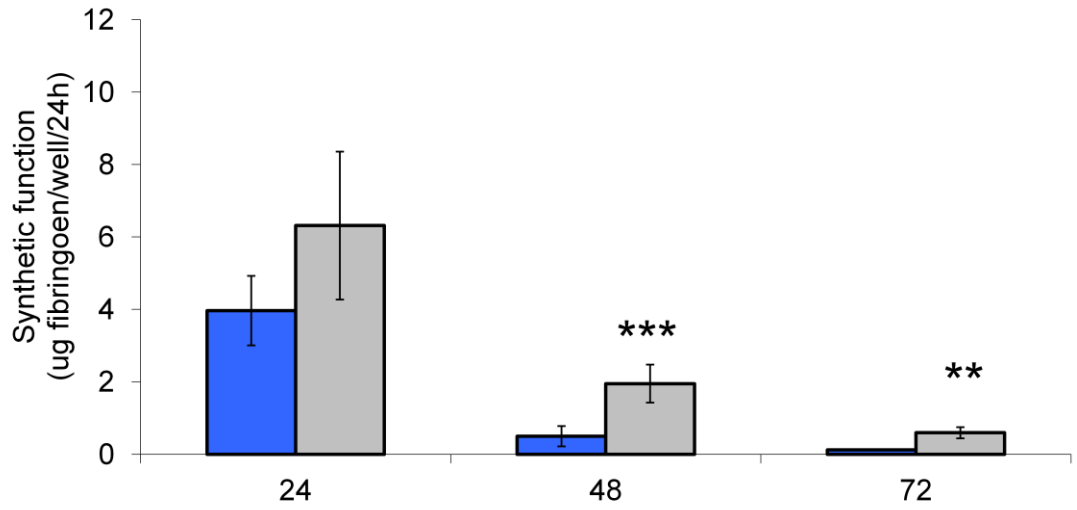


**Figure 7-27. Synthetic function of PHH cryopreserved with or without fructose in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (grey bars) fructose using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. . Time of function assay is indicated on the x axis. Results from 1 liver (BS 95) are shown. Data are averages of n=5 wells +/- SD. **p<0.01, ***p<0.005 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

7.4.7.4. Cytochrome P450 activity

Cytochrome P450 activity was more than doubled at both time points measured in PHH cryopreserved with fructose cf. those cryopreserved in standard CPA media. This was statistically significant at both time points (Figure 7-28).

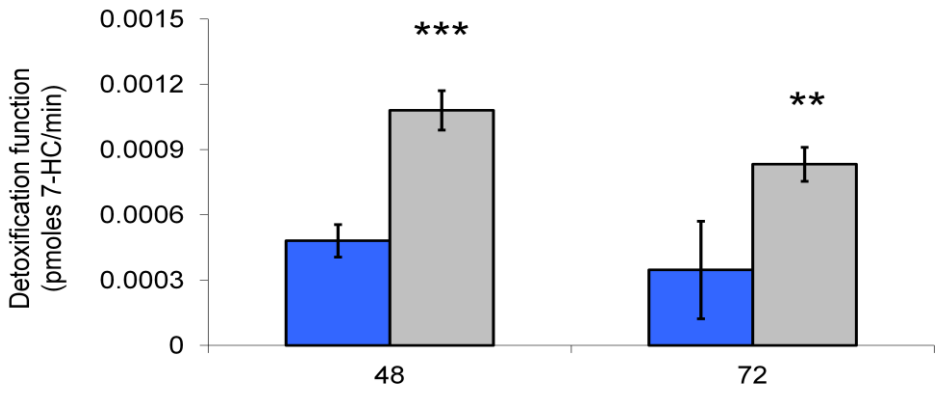


**Figure 7-28. Detoxification function of PHH cryopreserved with or without fructose in post-warming cultures.**

**Cryopreserved PHH were cooled in 12% DMSO/UW without (blue bars) or with (grey bars) fructose using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. CYP450 function was measured at 48 and 72 hours post-plating following 48 hour chemical induction. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD. **p<0.01, ***p<0.005 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

## 7.4.8. Are the effects of individual additives synergistic?

### 7.4.8.1. Attachment

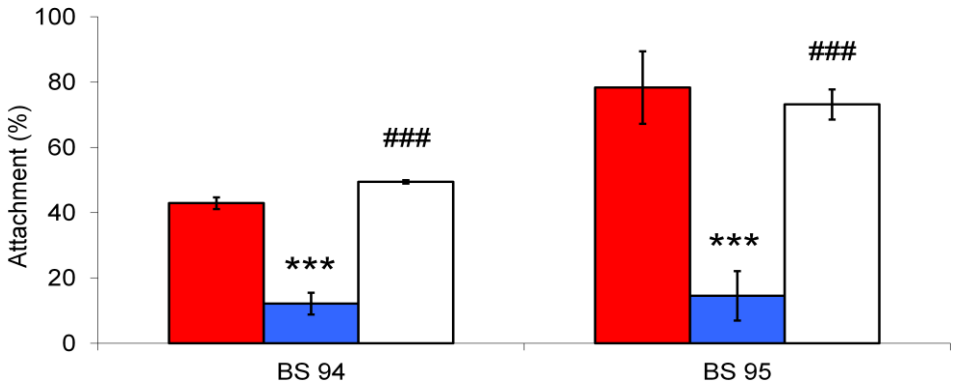


**Figure 7-29. Attachment of PHH cryopreserved using standard or optimal CPA mixes.**

**Cryopreserved PHH were cooled in 12% DMSO/UW (standard) (blue bars) or optimal CPA media (white bars) using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of attachment between fresh PHH (red bars) and PHH cryopreserved using either standard or optimal CPA media after overnight attachment. Results from 2 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. ***p<0.001 compared to fresh PHH from the same liver at equivalent time point. ###p<0.001 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

Attachment in PHH cryopreserved using standard CPA media was 13%, but was significantly improved in both livers when optimal CPA media was used to an average of 61% (49% in BS 94 and 73% in BS 95). Attachment in fresh PHH and PHH cryopreserved using optimal CPA media was not statistically different in either liver (Figure 7-29 on the previous page).

### 7.4.8.2. MTT Reduction

MTT reduction was significantly improved in both PHH at all 3 time points measured when optimal CPA media was used when compared to standard CPA media. On average, over the 2 livers at all time points, this improvement was approximately 6-fold. Despite this though, MTT reduction in PHH cryopreserved using optimised CPA media was still significantly lower than in fresh PHH, by nearly 5-fold.

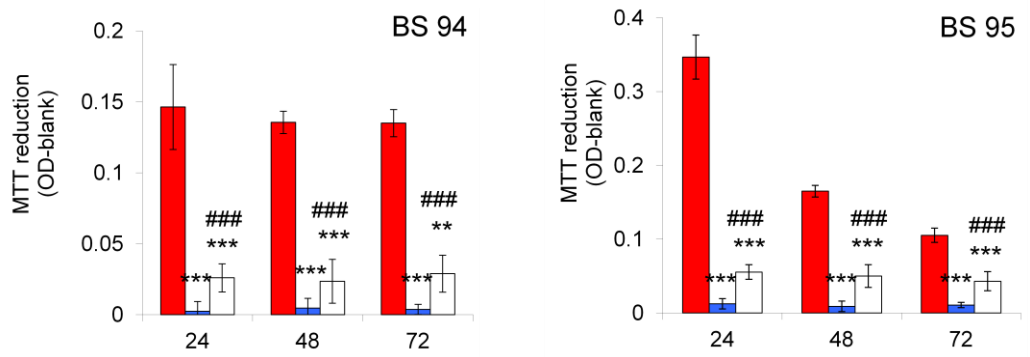


**Figure 7-30. MTT reduction of PHH cryopreserved using standard or optimal CPA mixes.**

**Cryopreserved PHH were cooled in 12% DMSO/UW (standard) (blue bars) or optimal CPA media (white bars) using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of MTT reduction between fresh PHH (red bars) and PHH cryopreserved using standard or optimised CPA media up to 72 hours post-plating. Time of assay is indicated on the x axis. Results from 2 separate livers are shown. Data from individual livers are averages of n=5 wells +/- SD. **p<0.005, ***p<0.001 compared to fresh PHH from the same liver at the equivalent time point. ###p<0.001 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

### 7.4.8.3. Protein Synthesis and Secretion

Protein synthesis and secretion was improved in PHH cryopreserved using optimal CPA media compared to PHH cryopreserved using standard CPA media. Over the 3 time points measured, this improvement increased with continued culture: at 24, 48 and 72 hours post-cryopreservation, increases in protein synthesis and secretion were 3-, 7- and 28-fold respectively. Despite this improvement in cryopreservation protocol, though,

even with optimal CPA media, protein synthesis and secretion remained significantly lower than from fresh PHH.

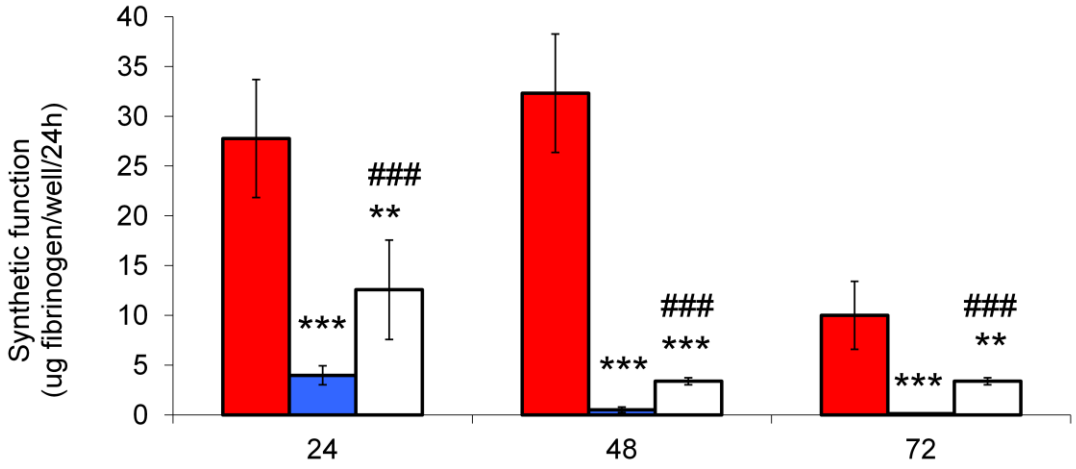


**Figure 7-31. Synthetic function of PHH cryopreserved using standard or optimal CPA mixes.**

**Cryopreserved PHH were cooled in 12% DMSO/UW (standard) (blue bars) or optimal CPA media (white bars) using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of protein synthesis and secretion between fresh (red bars) and PHH cryopreserved using standard or optimal CPA media up to 72 hours post-plating. Time of assay is indicated on the x axis. Results from 1 liver, BS 95, are shown. Data are n=5 wells +/- SD. **p<0.01, ***p<0.005 compared to fresh PHH from the same liver at the equivalent time point. ###p<0.001 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

### 7.4.8.4. Cytochrome P450 Activity

Cytochrome P450 activity was significantly improved using optimal CPA media compared to standard CPA media at both time points measured by over 3-fold at 48 hours and over 4-fold at 72 hours. Nonetheless, this improvement still resulted in a significantly decreased (32-fold) CYP450 function when compared to fresh PHH.

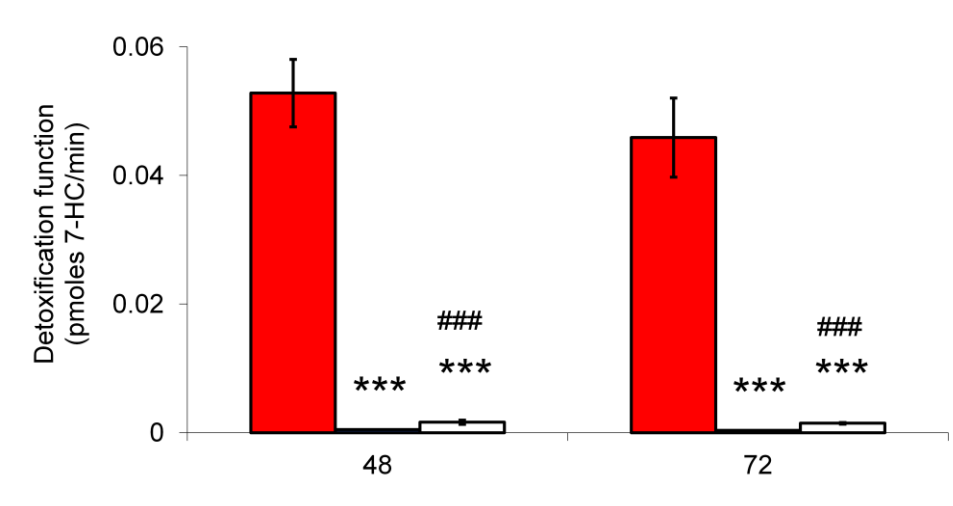


**Figure 7-32. Detoxification function of PHH cryopreserved using standard or optimal CPA mixes.**

**Cryopreserved PHH were cooled in 12% DMSO/UW (standard) (blue bars) or optimal CPA media (white bars) using a multi-step cooling profile (Diener et al. 1993) and warmed rapidly using a 37°C waterbath. Comparison of CYP450 function between fresh (red bars) and standard and optimally cryopreserved PHH at 48 and 72 hours post-plating following 48 hour chemical induction. Time of assay is indicated on the x axis. Results from 1 liver (BS95) are shown. Data are averages of n=5 wells +/- SD. ***p<0.001 compared to fresh PHH at the equivalent time point. ###p<0.001 compared to PHH cryopreserved using standard CPA media from the same liver at the same time point.**

## 7.5. Discussion

Cryopreservation of primary human hepatocytes yielding high numbers of fully functional cells would be extremely useful for studies of drug metabolism and toxicology models but also present an alternate cell source for a BAL. Whilst many groups have attempted to cryopreserve PHH, reported success varies dramatically.

The reasons for this are complex and some have been described earlier in this thesis. Briefly, as before, the time following cryopreservation at which recovery is assessed is likely to be influential. In many instances of previous publications, recovery is assessed immediately post-thaw which means that any latent injury may not yet be expressed, resulting in an overestimate of cell recovery. Here, recovery was assessed at 24, 48 and 72 hours post-cryopreservation which, hopefully, is representative of the true recovery of PHH in this study and is an appropriate time frame to observe in terms of intended usage of a BAL.

Secondly, as described earlier in this thesis, the assays used to measure recovery may result in different perceived outcomes. A wide range of assays are typically employed to assess hepatocyte recovery and include membrane integrity assays (typically trypan blue) and functional assays such as ammonia clearance and/or urea production, protein secretion and CYP induction (Stephenne et al. 2010). Trypan blue, or similar assays, typically overestimate functional cell recovery and so assays linked to specific metabolic processes give a better indication of cell status and are also relevant when considering use of PHH within a BAL where replacement of liver function is the overall aim. Here, recovery was assessed using cell attachment, MTT, protein secretion and CYP450 function in the hope that a more realistic view of PHH recovery after cryopreservation was achieved.

Finally, and irrelevant to ELS but relevant to PHH, is the cell source and method used to isolate the cells from the tissue section. Here, donor age varied greatly and most had undergone chemotherapy prior to resection which may be detrimental to PHH. This may be further complicated by the timing of stopping chemotherapy prior to resection. A strong correlation between viability pre-freeze (post-isolation) and post-freeze has previously been demonstrated. Viability post-isolation assessed using trypan blue was 46-77%. Similarly, a (less strong) correlation has been demonstrated between pre-freeze and post-cryopreservation PHH attachment in a similar culture format. This effect was not observed here but the numbers of separate livers assayed here are

considerably smaller than the 40 livers used in the earlier reported study (Terry et al. 2005) which may well account for the observed differences.

Whilst it is difficult to directly compare results found here with others due to the reasons described above, these results are generally in agreement with others where an identical cooling profile and CPA mixture was used. Cryopreserved PHH typically display reasonably good attachment but the function of these surviving PHH is low when compared to a fresh equivalent (Terry et al. 2005). Unlike ELS consisting of HepG2s, PHH are a heterogeneous population of individual cells and this is most visually apparent from micrographs of PHH assayed for MTT reduction where some cells appear very active whilst others do not, despite both cohorts being attached. This perhaps indicates that a sub-population of PHH are less susceptible to the effects of cryopreservation. This phenomenon has been observed previously in PHH but the reasons remain unclear although cell size (Alexandre et al. 2002) and *in situ* location within lobules (Moore & Gould 1984) have been suggested.

These results, and others, clearly show that standard cryopreservation techniques utilised to cryopreserve PHH are currently insufficient for a BAL but also demonstrate that there is great potential for improvement. A "baseline" recovery has also been established for the purposes of this chapter. Given that a number of additives have been shown to be beneficial for cryopreservation of ELS in previous chapters, these were tested for efficacy during cryopreservation of PHH.

The addition of cholesterol consistently improved ELS recovery following cryopreservation as assessed by viability, cell number and function (Chapter 3). In PHH, the addition of cholesterol was seemingly less effective. Attachment was improved in two of the livers but made no difference in a third and decreased attachment in a fourth. Similarly, MTT reduction was improved in half of the livers but unaffected in the other half by the inclusion of cholesterol. Both protein synthesis and secretion and CYP450 function were approximately doubled at the time points measured by using cholesterol-modified CPA media but these functional results were both from a single liver, BS 95.

Attachment of BS 95 was not improved by including cholesterol during cryopreservation, whereas the functional data from the same liver shows that total function was improved. This suggests that the function of the attached cells was improved using cholesterol, even though cholesterol alone was insufficient to increase attachment. This could be an indication that hepato-specific function of PHH (and

ELS) is more subtly affected following cryopreservation than attachment or general metabolism (MTT reduction) which is in agreement with previous findings (Chapter 3) relating to ELS and also that of other groups (Kunieda et al. 2003; Sosef et al. 2005).

Overall, the effect of cholesterol during cryopreservation of PHH does appear to be positive but is not as effective for cryopreservation of PHH as for ELS. The cooling profile used here was designed for PHH and does include a shock cooling step designed to trigger nucleation (i.e. to limit supercooling) which demonstrates that PHH are sensitive to supercooling (Diener et al. 1993). Cholesterol can limit supercooling further but here, it seems that PHH are not so sensitive to the effects of supercooling (and so improvements in recovery are not as great cf. ELS). The reasons for this are likely to result from the differing structural organisations. ELS are interconnected multicellular systems and water transport characteristics differ from that of single cell suspensions. Previous reports, one using a rotating cell culture system to form hepatocyte spheroids (Korniski et al. 1999) and another using collagen sandwich culture to form continuous hepatocyte monolayer cultures (Yarmush et al. 1992) both show that water permeability is decreased when hepatocytes are connected. In turn, this will increase likelihood of IIF (as interconnected cells are less able to dehydrate on a kinetic basis) and cell death. Here, no direct comparison has been made between hepatocyte suspension and interconnected hepatocytes but this mechanism of injury does apply to hepatocytes and so the argument above offers a plausible reason for the reduced efficacy of cholesterol for cryopreservation of PHH suspensions cf. ELS (i.e. the risk of IIF was low in any case for single cell suspensions during slow cooling).

In Chapter 4, 1K1 was demonstrated to protect ELS from cryopreservation-induced apoptosis. 1K1 has also previously been shown to protect PHH from apoptosis caused by Fas ligand. As apoptosis has previously been shown to occur following cryopreservation of mouse (Fu et al. 2001), pig (Matsushita et al. 2003; Yagi et al. 2001) and rat (Fujita et al. 2005) hepatocytes, it was hypothesised that apoptosis occurs in human PHH as a result of cryopreservation but may be reduced using 1K1.

It is not fully understood why apoptosis occurs in hepatocytes following cryopreservation but suggested causes include freeze-thaw injury (and in particular mitochondrial damage) and oxidative stress. Both of these are likely to cause damage in ELS too and have been discussed in previous chapters. However, specific to PHH here, is the concept of anoikis or "homelessness". Anoikis occurs in many epithelial cell types, including hepatocytes, and describes when detachment of cells from either each

other and/or surrounding supporting matrix and can result in apoptosis and decreased cell viability (Zvibel et al. 2002). Anoikis is likely to occur following PHH isolation from tissue here which may, in part, account for the observed apoptotic response.

Regardless of cause, apoptosis (indicated by caspase activation) has been shown to occur in PHH following isolation and cryopreservation. In this study, however, this was reduced using 1K1, an engineered form of HGF. Only one study could be found investigating the use of a growth factor (either native or engineered) to reduce apoptosis following cryopreservation: Shin et al. demonstrated that vascular endothelial growth factor could be used to reduce apoptosis (measured by caspase activation, and Annexin V and propidium iodide staining) in rat granulosa cells (Shin et al. 2006). However, a more common approach is to directly inhibit apoptosis using caspase inhibitors such as Z-VAD-FMK. This has previously been reported to successfully decrease apoptosis and increase viability in cryopreserved hepatocyte cultures (Fujita et al. 2005; Matsushita et al. 2003; Yagi et al. 2001).

As well as reducing apoptosis, 1K1 improved attachment in all livers (by varying amounts), and there was a non-significant trend to increased MTT reduction and protein synthesis at all time points and CYP450 function was unaffected. The effect of 1K1 on PHH function was only measured in BS 95 so it is difficult to suggest reasons as to why protein synthesis and secretion was improved but not CYP450 function. Attachment of BS 95 was only very slightly improved by 1K1, to a lesser degree than the other livers, which suggests that for some reason, PHH from BS 95 underwent less apoptosis following cryopreservation than other livers or that PHH from BS 95 were not as responsive to 1K1.

Despite the variation in results here, it seems that PHH do undergo apoptosis following cryopreservation but that this may be reduced using 1K1, resulting in improved attachment and metabolic viability. Oxidative stress is often suggested as a reason for initiation of apoptosis and has previously been implicated to occur in ELS (Chapter 4) and so the use of antioxidants was investigated in this study

Oxidative stress has often been reported as an injury mechanism that occurs during cryopreservation that results in decreased cell viability and function. This is true for many cell types, notably sperm (Tatone et al. 2010) but also for hepatocytes (Illouz et al. 2008). In Chapter 4, ELS were also demonstrated to suffer the effects of oxidative stress and that these may be reduced using antioxidants during cryopreservation.

Many reports describe occurrence of oxidative stress during cold storage (with or without freezing) in liver cells and tissue. Suggested causes of oxidative stress include reductions in cellular glutathione content (Stevenson et al. 2007; Vreugdenhil et al. 1991), increased cellular biologically reactive iron content (which goes on to take part in Fenton chemistry) (Wyllie et al. 2003) and osmotic stress (McCarthy et al. 2010). Oxidative stress then results in decreased recovery following cold storage.

Much of the literature focuses on the effects of oxidative stress on sperm cells during cryopreservation. Catalase has been used to protect sperm from oxidative stress at concentrations similar or identical to the 500IU/ml used here (Fernandez-Santos et al. 2009; McCarthy et al. 2010; McCarthy & Meyers 2011). Similarly, vitamin E (hydrophobic Trolox) protects sperm from oxidative stress at 0.1mM (McCarthy et al. 2010; McCarthy & Meyers 2011) and at 0.05mM (Maia et al. 2010). In another study, 0.1mM Trolox protected rat hepatocytes from oxidative stress during cold storage (Arthur et al. 2008). These concentrations are lower than the 1.7mM used here. In Chapter 4, 1.7mM was demonstrated to be optimal for ELS and this was the concentration chosen to apply to PHH in this study.

Here, cryopreservation caused a large increase in lipid peroxidation but this injury was reduced back to levels comparable to that of fresh PHH when antioxidants were employed during cryopreservation. These results were from only one liver and were not statistically significant. Lipid peroxidation was normalised to total protein as attachment was variable between the fresh and cryopreserved cohorts of PHH. The variation in the results was caused by difficulties in quantification of protein content in PHH cryopreserved in standard CPA media (i.e. it was very low, on the limit of detection for the assay). Despite this, it seems that oxidative injury in PHH can be reduced using antioxidants, although further testing is required to confirm this.

Antioxidants improved attachment, MTT reduction (in one liver) and protein synthesis and secretion although did not improve cytochrome P450 activity (as for 1K1). These results were not always statistically significant although a general trend of improved recovery was seen. This suggests that although PHH are prone to injury from oxidative stress, it is perhaps not a major contributor to loss of viability and/or function. Alternatively the dosage of catalase and Trolox tested here may be inappropriate for PHH (although they were optimal for ELS). In addition, the challenges for antioxidant therapy include delivering correct doses to specific cellular compartments (such as

damaged mitochondria). Adding antioxidants to (CPA) media may not achieve real therapeutic doses, and this is a complex issue which requires in depth study.

FFP improved attachment, MTT reduction and protein synthesis and secretion of PHH although, again, CYP450 activity was unaffected. FFP appeared to improve recovery to a greater degree and more consistently than the other additions, the results of which are given above. It is difficult to discern which component(s) of FFP result in the improved recovery of PHH although it is likely that the mechanisms of protection are similar to that of serum, such as membrane stabilisation, buffering capacity or supply of growth factors. Serum (normally bovine) is routinely used in cryopreservation of human hepatocytes (Li et al. 2009; Stevenson et al. 2004).

Again, it seems that although FFP consistently improved recovery of PHH; however, the degree by which improvements were observed varied between livers. For example, in BS 94, attachment of PHH was increased by 1.5-fold by FFP but in BS 95, attachment of PHH was more greatly improved by nearly 4-fold. Similarly, improvements in attachment do not necessarily match improvements in MTT reduction or function and variation between livers exists here too. In BS 95, whilst cell attachment was quadrupled, MTT reduction and protein synthesis and secretion were only doubled and CYP450 activity was unaltered. Conversely, in BS 94, where attachment was only slightly improved (albeit significantly) MTT reduction was improved by approximately 3-fold. Again, these results demonstrate and further confirm that inter-liver/isolation variations have major impacts on overall PHH functionality.

The addition of fructose during cryopreservation appeared to be beneficial for PHH cryopreservation. Improvements in attachment, MTT reduction, protein synthesis and secretion and CYP450 activity were seen, although all of these results came from a single liver, BS 95. In another liver, BS 90, no improvement in MTT reduction was observed when PHH were cryopreserved with 300mM fructose, although it was not detrimental. Again, this demonstrates variability between different livers, isolations and PHH.

Fructose has previously been reported to improve recovery of PHH in post-thaw cultures as measured by attachment efficiency and albumin secretion, although viability was unaffected. Whilst the exact mechanism of protection remains unknown, the authors suggest a number of possible modes of action including formation of nicotinamide adenine dinucleotide phosphate which can react to form glutathione to

protect against oxidative stress, and subsequent apoptosis. In addition, fructose can be readily metabolised, if needed by glycolysis to boost adenosine triphosphate (ATP) cellular content under aerobic/hypoxic conditions (Terry et al. 2005). Hypoxia may occur during transport, isolation or cryopreservation (Clavien et al. 1992). ATP cellular content has previously been reported to be decreased in PHH after cryopreservation due to altered mitochondria (Stephenne et al. 2007) and so fructose may provide a means of ameliorating this. The ability to support ATP production via glycolysis may be beneficial in cryopreservation where mitochondria may be injured and thus cannot support normal aerobic metabolism in the early post-thaw period. These proposed mechanisms of injury seem not only feasible but also well-aligned with those identified earlier in this chapter and thesis.

Having determined that the effects of cholesterol, antioxidants, 1K1, FFP and fructose were separately beneficial as individual additions to CPA media, this section of work aimed to find out if the effects of these were additive. Individually, these components were insufficient to improve the standard CPA media to allow recovery of PHH to a degree that they could be considered as essential components for use within a BAL (or in metabolism or toxicology studies).

From my results, it now appears that these components do have a synergistic effect. Attachment was markedly improved, to the point where attachment efficiencies between fresh and optimally-cryopreserved PHH was not statistically significant. This was not achieved using any single additional component, although FFP did show a major effect on attachment when used as a single additive.

Similarly, MTT reduction, protein synthesis and secretion, and CYP450 activity were also greatly improved. Optimal improvements in MTT reduction were achieved using FFP where a 2-fold improvement was achieved, lower than the 6-fold improvement seen using optimal combined additives CPA media. Protein synthesis and secretion was doubled using the components individually but this was greatly improved using optimal CPA media to 3-, 7- and 28-fold at 34, 48 and 72 hours post-thawing respectively. Futhermore, CYP450 activity was only doubled using cholesterol and fructose individually but was tripled using optimal combined additives CPA media.

This suggests that the effects of the components seen individually are also additive to a degree. As some of the components have "overlapping" modes of protection (i.e. 1K1, antioxidants, and to a less degree fructose, protect against apoptosis), it is not entirely surprising that their effects are not entirely cumulative. However, the greatest overall

improvement was observed using the optimal combined additives CPA media. Despite this, the function of these cryopreserved PHH still remains low compared to fresh PHH indicating that recovery is still some way short of producing optimally-functional PHH post-thaw in a reliable fashion.

For convenience, the results from all these studies are summarised below in Table 7-3 below.

**Table 7-3. Summary of results from this chapter showing improvements in recovery achieved using components individually or cumulatively.**

| | **Attachment** | **MTT reduction** | **Protein synthesis and secretion** | **Cytochrome P450 activity** |
|---|---|---|---|---|
| **Cholesterol** | 1.5-fold ↑ | <1.5-fold ↑ | 2-fold ↑ | 2-fold ↑ |
| **1K1** | 1.5-fold ↑ | 1.5-fold ↑ | 2-fold ↑ | No effect |
| **Antioxidants** | 2-fold ↑ | No effect | 2-fold ↑ | No effect |
| **FFP** | 2.5-fold ↑ | 2-fold ↑ | 2-fold ↑ | No effect |
| **Fructose** | 1.25-fold ↑ | 1.5-fold ↑ | 2-fold ↑ | 2-fold ↑ |
| **Optimal** | 4-fold ↑ | 6-fold ↑ | 3-, 7, 28-fold ↑ | 3-fold ↑ |

## 7.6. Conclusions

The aim of this Chapter was to determine if the same additives that improve cryopreservation of ELS, also improve cryopreservation of PHH. Additives were tested individually and together. In agreement with the findings of others, these results demonstrate the need to assay cells for the function which is relevant for the desired application. This chapter also shows how variable PHH are and a large limitation to this study is the small numbers of livers which could be studied in the available time.

Cholesterol resulted in a small improvement in recovery of PHH. However, this was not as large as the improvements observed in ELS which likely results from differing culture formats. ELS are multicellular and exhibit different osmotic characteristics which make them more vulnerable to the damaging effects of supercooling. PHH on the other hand, appear less sensitive to supercooling.

1K1 acted as an effective anti-apoptotic and caspase activity was reduced in post-thaw cultures resulting in improved recovery, as for ELS.

Antioxidants were also effective for PHH, reducing lipid peroxidation and again improving recovery in post-thaw cultures.

FFP also improved recovery, more markedly than for ELS and attachment in particular was greatly improved. Perhaps, membrane stabilisation is more important for PHH where attachment to collagen is required for survival. Conversely, ELS are already entrapped within alginate and so this may be less relevant for ELS.

To summarise, all these components were found to be beneficial individually, and synergy was seen when used in conjunction. This demonstrates that PHH undergo the same damaging effects caused by cryopreservation as ELS (albeit by different amounts). This suggests that these techniques and approaches may be transferable to improve cryopreservation, at least, other liver-derived cells, and may be generally applicable to other organ-derived cells (such as islets of Langerhans from pancreas).

# CHAPTER 8

# General Discussion: Summary of Findings and Future Work

The overall aim of this thesis was to optimise a cryopreservation protocol for encapsulated liver cell spheroids (ELS) that would eventually comprise the cellular component of a bioartificial liver (BAL) intended for treatment of acute liver failure (ALF). ALF has an extremely rapid and unpredictable onset but ELS require up to 10 days to reach performance competency (i.e. until they are available in sufficient number and possess sufficient function) (Coward et al. 2009) and so an optimised protocol for cryopreservation of ELS is required. This protocol should be optimised to provide the greatest number of functionally viable ELS.

## 8.1. Approaches to assess cryopreservation protocol efficacy

Ultimately, this cryopreservation protocol for ELS aims to replace total liver function in patients with compromised liver function. The liver is an extremely complex organ which performs a wide range of functions. Prior to the start of my work, HepG2 cells were selected as the cell of choice for this BAL as they are available in sufficient number (unlike primary cells), proliferate well in culture and their function is upregulated in 3D spheroids, to levels similar to that of primary cells *in vivo* for some functions, namely synthetic (Coward et al. 2009).

To assess efficacy of cryopreservation protocols for ELS, 3 main indicators were selected: viability, used here to describe membrane integrity balanced by cellular metabolism; cell number; and function, normally assessed from synthesis of hepato-specific function. The viability assay used here was useful as it allowed rapid assessment of ELS over time and moreover, correlated well with cell numbers and function, making it a robust method of assessing recovery of ELS. Moreover, the maintenance of membrane integrity in ELS is important to prevent transfer of cellular debris back to the patient. Cell numbers should also be maintained so that sufficient function can be provided but also again, to provide assurance that biomass from the BAL was not at risk of returning to the patient. Replacement of function however, remains the ultimate goal and so functional assays were also used throughout my study. As the synthetic function of ELS is approaching that of the liver *in vivo*, this was the functional assay of choice. Other liver-specific functions are either lacking

(detoxification of ammonia (Mavri-Damelin et al. 2008)) or depleted (cytochrome P450 function (Westerink & Schoonen 2007)), making measurement difficult in cryopreserved ELS where function is further reduced.

## 8.2. Characterisation of ELS following cryopreservation

### 8.2.1. Establishing baseline recovery

The first aspect of this thesis aimed at establishing a baseline recovery for ELS following conventional cryopreservation using a published method that had been optimised for cryopreservation of primary human hepatocyte (PHH) cell suspensions (Diener et al. 1993; Terry et al. 2006). Although this method was optimised for cryopreservation of PHH, alone, it was not suitable for ELS. Viability, viable cell numbers and function remained low indicating that this protocol could not be used for ELS, and that further optimisation was required.

### 8.2.2. Establishing timescale of recovery

Unlike some other cryopreservation protocols, cryopreservation of ELS requires that ELS recover rapidly in post-thaw cultures. Treatment of ALF should begin soon after diagnosis to improve clinical outcome and so performance-competent ELS should be provided to enable this. In other applications, cell death in post-thaw cultures is not necessarily problematic if, for example, a cell bank is being established so long as dead cells can be removed from culture so that they do not damage the surviving population. My data showed that ELS were not capable of sufficient recovery within a suitable timeframe to treat ALF using the initial protocol, that 24 hours post-warming was when most injury was apparent and that recovery period was initiated thereafter out to 72 hours post-warming.

## 8.3. Improving ELS recovery

### 8.3.1. Achieving control of nucleation event

One of the first observations made, and described in Chapter 3, was that significant supercooling was occurring using the starting cooling profile {Diener, 1993 7 /id}. Supercooling is known to be damaging to cell suspensions and, as a result, methods have been developed to avoid/limit this event. The most commonly used method is that of seeding, where nitrogen-cooled forceps are touched to the side of the freezing vessel to induce localised deep supercooling thus triggering nucleation. However, this

approach is unsuitable for cryopreservation of ELS where cryopreservation of large volumes of biomass will eventually be required as it would be difficult to remove heat, even locally, from a large mass.

Heterogeneous nucleators provide an alternative method of triggering nucleation, limiting supercooling. In my study, cholesterol was confirmed as an effective nucleator (Head 1961) and recovery of ELS was greatly improved when it was included during cryopreservation. The approach of using cholesterol could be also readily transferred to larger volumes, making it an appropriate method for this application.

Supercooling damages cells as it results in the application of unintended rapid cooling rates to cells, which reduces the time in which cellular dehydration can occur, increasing the likelihood of intracellular ice formation (IIF). IIF is often lethal and so supercooling must be avoided or, at least, limited. Cryomicroscopy was used to confirm that this was the injury mechanism that cholesterol ameliorated. Without cholesterol, there was evidence of supercooling, followed by rapid nucleation and formation of ice within spheroids. That ice can propagate between cells is a known theoretical possibility and has since been demonstrated experimentally (Acker et al. 2001; Armitage & Juss 2003; Berger & Uhrik 1996). For ELS, this means that ice formation in any portion of the spheroid may propagate throughout the remainder of the spheroid, which is likely to be lethal. Conversely, when cholesterol was present, supercooling did not occur to the same degree, ice formation was more gradual and the alginate itself provided a protective barrier to the ice front and the alginate bead itself decreased in volume. Spheroid dehydration was not observed but there was no evidence of IIF, confirming cholesterol's mode of action.

### 8.3.2. Limiting apoptosis during cryopreservation

Whilst the use of cholesterol significantly improved ELS recovery, further improvements could be made, as recovery was still not complete. Necrosis and apoptosis have previously been recognised to occur following cryopreservation (Baust et al. 2002; Baust et al. 2000; Bilodeau 2003; Chaveiro et al. 2007). Whilst necrosis is likely to occur following gross physical disruption of the cells, apoptosis results from cell signalling and so strategies can be developed to try to limit the onset of apoptosis and this was tested in Chapter 4. Here, oxidative stress and hypothermia were tested as potential causes of apoptosis.

8.3.2.1. Hypothermic shock

In my study, attempts were made to upregulate expression of heat shock proteins (HSPs) so that hypothermia could be combated. Upregulation was by exposure of ELS to hyperthermia in line with other published data, but this approach did not convey further protection to ELS. This may be because the process of cryopreservation is too quick and that actually hypothermia for such a short time period is not damaging to ELS or it may be that HSPs may not be further upregulated since they are already upregulated in 3D cultures (Choudhury et al. 2003).

8.3.2.2. Oxidative stress

Oxidative stress is often implicated as an injury mechanism during cold storage both with and without ice formation (Bilodeau et al. 2001; Cerolini et al. 2000; Forrest et al. 1994; Oh & Lim 2006; Paudel et al. 2010). Here, antioxidants were tested for ability to improve ELS recovery following cryopreservation and, indeed, they were effective. In order to prove their mode of action, I looked at production of reactive oxygen species (ROS) and lipid peroxidation as an indicator of oxidative damage. What I found was that hypothermia both with and without freezing resulted in increased levels of ROS and subsequent oxidative damage. Both could be reduced using antioxidants either before or after cryopreservation but this reduction was only statistically significant when antioxidants were administered before and after cryopreservation, indicating that protection from oxidative stress was required throughout the entire process.

8.3.2.3. 1K1

Hepatocyte growth factor (HGF) is known to possess anti-apoptotic properties but is expensive and difficult to produce. 1K1 is an engineered version of HGF but is cheaper and easier to produce and so, was tested in my study for prevention of apoptosis resulting from cryopreservation (Ross et al. 2009). Firstly, its efficacy was tested against apoptosis-causing staurosporine (without freezing) and I found that it limited caspase activity at levels that were no different to untreated cells. Following this, I tested its efficacy within a cryopreservation protocol and found that caspase activity was reduced (although not eliminated entirely) and that this resulted in improved viability, viable cell numbers and total function in post-thaw cultures.

By combining cholesterol, 1K1 and antioxidants, good ELS recovery was achieved, that would be suitable for use within a BAL.

## 8.4. Scale-up

Having optimised the cryopreservation protocol for ELS on a small scale, the cryopreservation then required scaling up to achieve sufficient biomass for treatment of an adult patient suffering with ALF. There were two aspects to this: firstly, whilst undertaking this work, the culture process for ELS was significantly improved meaning that far more cell-dense ELS could be achieved, and the effect of this on cryopreservation needed to be determined; secondly, ELS needed to be required in far greater volumes. Until this point, ELS had been cryopreserved in 0.25ml volumes in 1.8ml cryovials but, clearly, this would be impractical for the final BAL.

### 8.4.1. Effect of ELS cell density on recovery during cryopreservation

In Chapter 5, I found that as cell density within ELS increased, recovery following cryopreservation decreased. This was true for ELS encapsulated using both the Inotech syringe pump system and cultured in static conditions and also for ELS encapsulated using the JetCutter system that were cultured in the fluidised bed bioreactor (FBB). Cell-dense ELS contain larger spheroids, each comprising more cells than less cell-dense ELS. This means that these spheroids are harder to dehydrate and are more prone to injury from IIF (resulting from supercooling or otherwise). In Chapter 3, I demonstrated that cholesterol along with the selection of cooling rate of ~2°C/minute was sufficient to reduce IIF incidence, and that slower cooling rates were detrimental to ELS recovery. However, in Chapter 5, for cell-dense ELS a slower cooling rate of 0.3°C/minute was not detrimental but instead resulted in better recovery. This supports the hypothesis that cell-dense ELS require more time for cellular dehydration to avoid often-lethal IIF.

### 8.4.2. Cryopreservation of ELS in greater volumes

#### 8.4.2.1. In cryobags

When ELS were cryopreserved in cryobags, recovery did not differ from those ELS cryopreserved in cryovials, indicating successful upscaling of the cryopreservation process to this volume. Temperature profiles of the two vehicles were similar during cooling and cholesterol acted as an effective heterogeneous nucleator in both. Similarly, during thawing, rapid warming was readily achieved. This was a successful approach as although the length and width of the vessel increased greatly, the depth of

the cryobags remained similar to that of the cryovial. This is a significant finding as, in theory, a very much larger volume could be cryopreserved as long as the dimensions were appropriate (i.e. limited to 3mm in one).

#### 8.4.2.2. In FBB culture chamber

If ELS could be cryopreserved within a disposable FBB culture chamber, a truly off-the-shelf BAL device could be provided. When ELS were cryopreserved in the FBB culture chamber, temperature profile differed from both the cryovials and cryobags: no supercooling was apparent at all; and the culture chamber took far longer to cool to the desired temperature. However, the rate achieved was not slower than 0.3°C/minute and so it seems likely that ELS could survive the cooling process.

Achieving a rapid warming rate was more complex though. Using the same protocol as for cryovials and cryobags (i.e. immersion into a 37°C waterbath) was ineffective and the culture chamber took nearly 2 hours to thaw. As a result, viability of thawed ELS was far lower than either the cryovials or cryobags and by 48 hours post-warming, only a very few cells stained positive for fluorescein diacetate (FDA). Clearly, this is not suitable for use within a BAL.

In order to attempt to achieve a more rapid warming rate, the culture chamber was modified to include tubing through which a warmed liquid could be pumped to speed up the thawing of the biomass. This approach was demonstrated to be successful in a test run without ELS and thawing was complete within approximately 10 minutes. However, when this system was tested on ELS, the tubing failed and so the effect of a "rapid thaw" in the culture chamber containing a large volume of ELS could not be assessed.

## 8.5. Regulatory considerations

Given that the BAL will eventually be used for treatment of human patients suffering from ALF, the entire BAL production process (including cryopreservation and thawing) will have to meet regulatory requirements.

Although this is largely beyond the scope of this work, in Chapter 6, I demonstrated that a number of aspects of cryopreservation could be carried out whilst still complying to these regulations (e.g. GMP-compliant culture media are suitable for cryopreservation of ELS).

One of the most interesting findings was regarding the use of liquid nitrogen for both controlled cooling and storage of ELS. Avoiding nitrogen entirely would be preferable as it needs to be of pharmaceutical grade, which is costly, and it requires specialist facilities and training of staff etc. Controlled cooling of ELS using a cryogen-free cooler was possible and, importantly, with similar functional recoveries to those ELS cryopreserved using a traditional nitrogen-cooled controlled rate freezer (CRF) (Massie et al. 2011). However, long-term storage of ELS at temperatures above the glass transition temperature of the cryopreservation media (CPA) was not suitable for ELS. Storage at -80°C resulted in decreased functional recovery after as little as one month, which is an important finding and should be considered when, in the future, a cold-chain delivery strategy is developed for the BAL.

## 8.6. Primary human hepatocytes

PHH do represent a possible alternative to HepG2 cells for use within a BAL, although, it is more likely that any viable PHH would be preferentially utilised for transplantation. In any case, cryopreservation of PHH is potentially useful for a BAL, but also for drug metabolism studies.

Whilst the main scope of this work was focused on cryopreservation of ELS, if the same techniques that proved successful for ELS also proved successful for PHH, this provides confidence in the results and suggests that other (liver) cell types may also benefit from the specific additions.

In Chapter 7, various approaches were tested and were largely successful and also synergistic. Although the approaches made were successful in terms of proving specific additives beneficial, the overall recovery of PHH remained very low when compared to freshly isolated PHH and the results, although interesting from a scientific point of view, could not be considered useful for clinical applications. In this case, these findings validate the use of ELS as the cellular component of our BAL.

## 8.7. Future work

If I were to continue this work further, I would focus on achieving better recovery in cell-dense ELS. Cell-dense ELS will be used in the final BAL and so developing a method of cryopreserving these is vital. It seems most likely that cryopreservation was unsuccessful for cell-dense ELS because insufficient time was allowed for ELS

dehydration. This could be confirmed visually using cryomicroscopy which could be used to observe IIF.

If IIF was observed, then the use of non-penetrating CPA could aid dehydration. Slower cooling rates could also be used. Moreover, as cholesterol is such an effective nucleator, removal or modification/shortening of the shock-cooling step could prove effective. In any case, shock-cooling is unlikely to be effective for large volumes of ELS and so, in any case, it would not necessarily convey any benefit but instead shortens the time for ELS dehydration.

Cryopreservation in large volumes also requires further work and so this could also be an area for future work, in particular the development of a method for rapid and reliable thawing. This is required to avoid recrystallisation and devitrification events. Although a method has been developed, it has not yet been tested with ELS and so it would be interesting to do so. However, this would require significant engineering knowledge and manufacturing capabilities.

The use of cholesterol proved extremely beneficial for ELS and so its effect on other multi-cellular systems could be investigated. Pancreatic islets are considered structurally similar to ELS and there is a clinical need for cryopreservation for these. Success could be measured using both viability (membrane integrity assays) but also functional tests (such as glucose stimulation index) (Schneider & Klein 2011).

It would also be interesting to further investigate the cell biology behind the proposed injury mechanisms. For example, elucidation of some of the differences or similarities between cold storage with and without freezing could offer some insight as to how to prevent onset of apoptosis more efficiently.

### 8.8. What others have achieved

When considering the outcome of my work, what has been achieved by others should be considered. As discussed more fully in Chapter 7, there is a vast amount of published literature on previous attempts to cryopreserve primary human hepatocytes for a BAL or otherwise. I would argue that not only is function too greatly reduced in PHH following cryopreservation using current cryopreservation protocols, PHH are unlikely to be available in sufficient number to comprise the cellular component of a BAL; instead, they would more likely be utilised as a whole organ for transplantation or for cell transplantation.

A few published papers talk specifically about the cryopreservation of animal hepatocytes specifically for use within a BAL. 2 papers discuss cryopreservation of porcine hepatocytes and a third discusses cryopreservation of rat hepatocytes. Whilst animal cells do have the advantage of being available in sufficient number, the risk of viral transmission (of for example, porcine endogeneous retroviruses) persists.

Nyberg et al. describe cryopreservation of porcine hepatocytes which are then formed into spheroids (with upregulated per cell function cf. monolayer cultures) using a rocking culture system over up to 5 days. When they compared per cell albumin production between fresh and cryopreserved cells, the function of the cryopreserved cells was 2% that of the fresh equivalent on day 1 and by day 5, function of the cryopreserved cells was reduced to 0.2% that of the fresh equivalent (Nyberg et al. 2005). Clearly, this is insufficient but in addition to this, the system is somewhat flawed in that 5 days is probably too long anyway to be useful in treatment of ALF.

More recently, Giri et al. describe cryopreservation of porcine hepatocytes that are sandwiched between 2 collagen layers intended to be part of a BAL. In that system, cells were cryopreserved 3 days after seeding before function was compared between the cryopreserved cells and fresh equivalent up to 14 days post-cryopreservation. What they found was that per cell albumin secretion was very low immediately post-cryopreservation but was completely recovered by day 14. Urea production was also reduced (by approximately 50% post-cryopreservation) but did not recover even by day 14. Finally, they looked at CYP450 function and again, function was reduced by nearly 75% in cryopreserved cells on day 3 after cryopreservation but by day 14, CYP450 function in cryopreserved cells was half that of the fresh equivalent (Giri et al. 2010). This approach was more successful than that of Nyberg et al. but again, the recovery period of the cells in post-thaw cultures is probably too long for treatment of ALF. However, this approach does have the advantage of providing an off-the-shelf product as the cells are cryopreserved in the same format as they would be used in in a clinical setting.

Finally, Son et al. discuss cryopreservation of rat hepatocytes that are then encapsulated into alginate/chitosan beads. The ammonia removal/urea production function of the cryopreserved cells was compared to the fresh equivalent and again, function was reduced immediately post-cryopreservation by ~40% but returned to levels similar to that of the control by 3 days post-cryopreservation, which is quicker than either of the 2

studies described above and may be quick enough for treatment of ALF (Son et al. 2006).

None of these papers though, talk about total function of the cryopreserved cells compared to the fresh equivalent. As argued earlier in this thesis, total function is very important as it is the most probable predictor of BAL success. In addition, these papers do not indicate cell number recovery following cryopreservation and a loss in cell number indicates potential for contamination of patient plasma with foreign cellular material. These papers highlight the continuing challenges in cryopreserving a biomass for a BAL.

## 8.9. What my current system can achieve

The current cryopreservation protocol allows for successful cryopreservation of ELS at lower cell densities with recovery close to levels of unfrozen ELS within a suitable timeframe for treatment of ALF.

However, cryopreservation of cell-dense ELS is not currently sufficient for use within a BAL but further work could certainly improve this further, using the strategies described earlier in this Chapter.

A promising finding is that the protocol can be upscaled as would eventually be required for the final BAL, indicating that successful cryopreservation of ELS is possible in large volumes.

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